PULMONARY TUBERCULOSIS IN HIV INFECTION IN TANZANIA

clinical and immunological studies

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dedication

To my children

In memory of my mother and father

Contents

Chapter 1

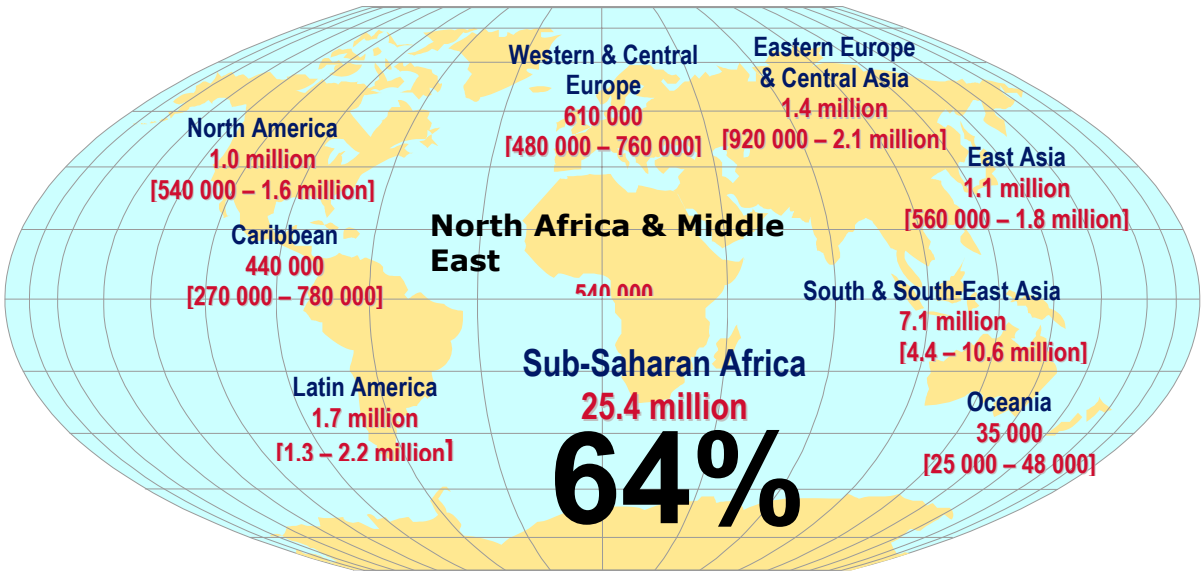
General introduction and outline of the thesis

INTRODUCTION

Tuberculosis: The HIV factor

Of the 40 million persons living with HIV/AIDS to-date, over 60 percent reside in sub-Saharan Africa, making it the most affected region of the world, **Figure 1** (1;2). HIV infection rate is still on the increase. In 2006 alone of the estimated world total of 4.3 million (3.6 – 6.6 million) new HIV infections, sub-Saharan Africa was estimated to have 2.8 million (2.4 – 3.2 million) adults and children infected with HIV. This was more than all other regions of the world combined together (2). The 2.1 million AIDS deaths in sub-Saharan Africa represented 72% of global AIDS death (2).

Figure 1. Adults and children estimated to be living with HIV/AIDS (UNAIDS, 2004)



In Tanzania in particular, at the end of 2005, the estimated number of adults and children who were living with HIV was 1.4 million (1.3 – 1.6 million), making Tanzania one of the most affected countries in the world

(2). It was projected that in Tanzania new infections and death will increase steadily to reach 250,000 and 120,000 cases per year, respectively in 2010, **Figures 2,3,4** (3).

Figure 2. Estimates and projections of HIV infection among adult Tanzanians from 1980 to 2010

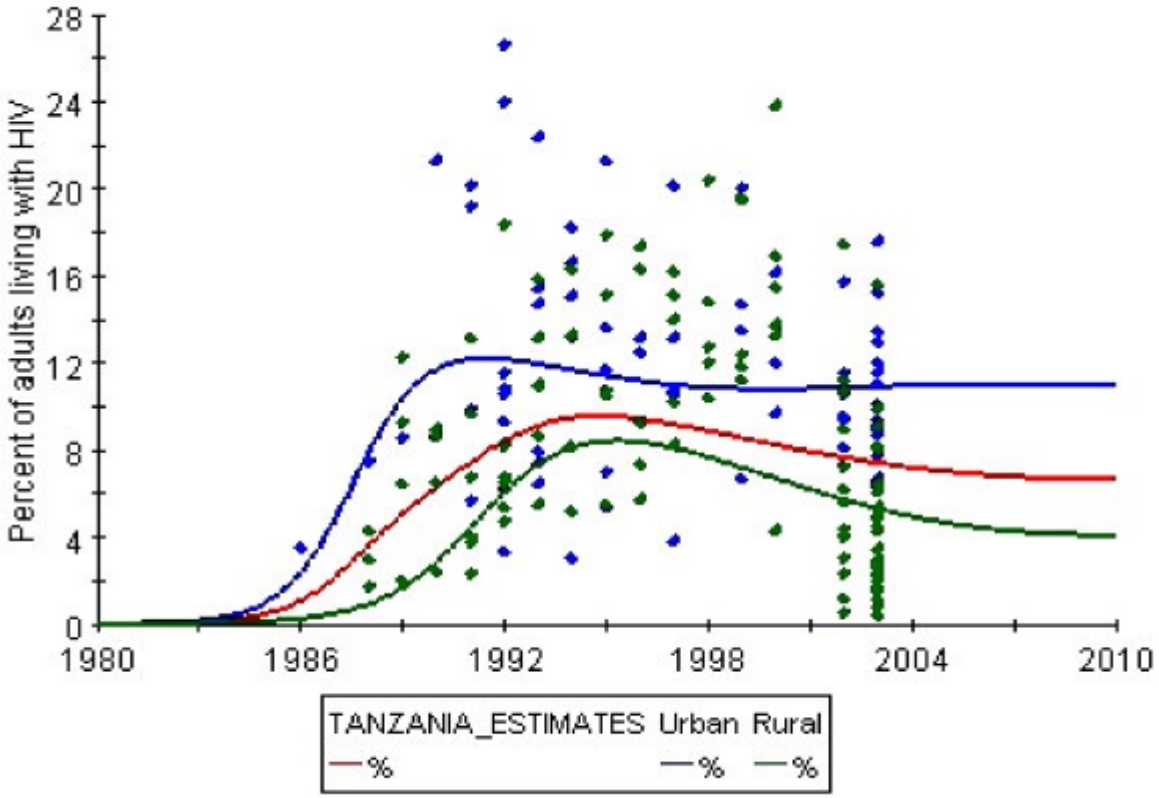


Figure 3. Estimates and projections of new cases of HIV infection among adult Tanzanians from 1980 to 2010

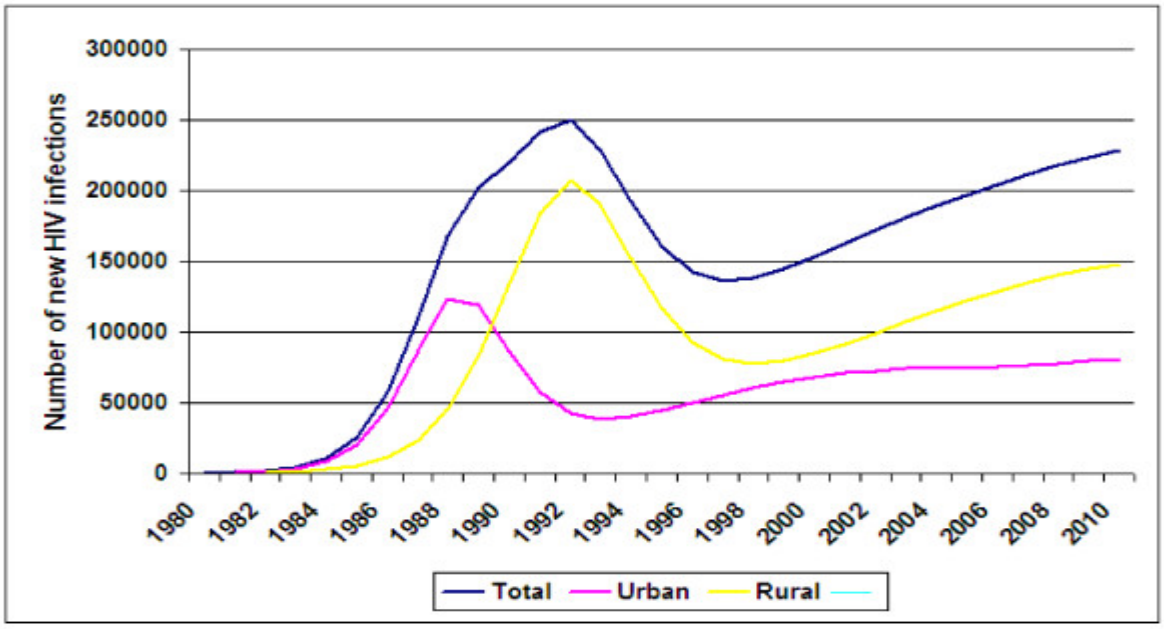
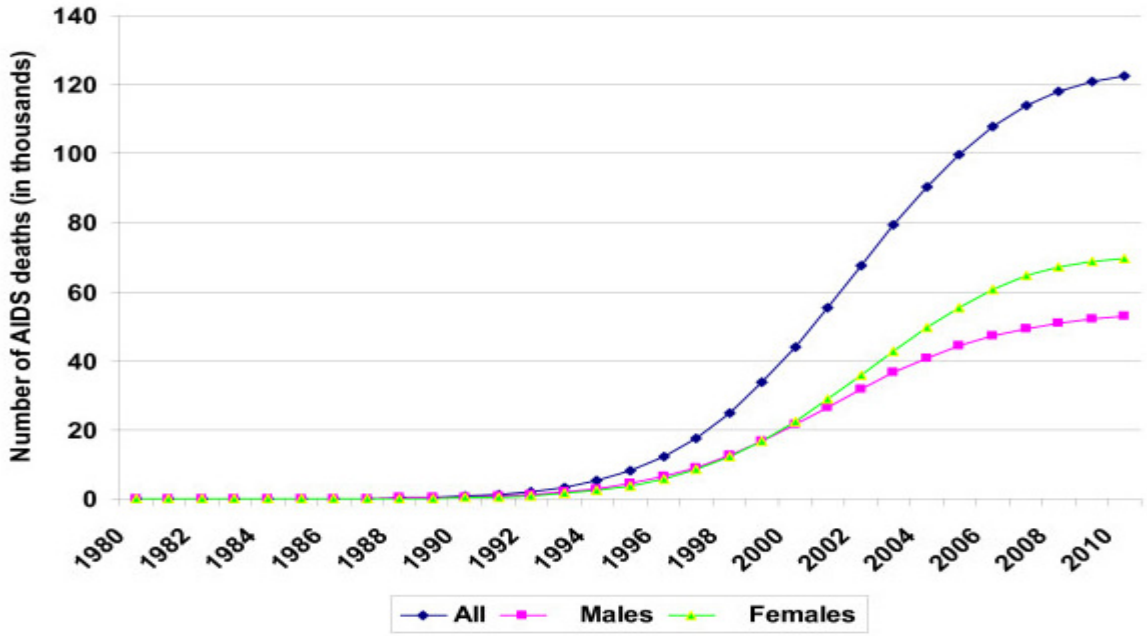


Figure 4. Estimates and projections of aids deaths among adult Tanzanians from 1980 to 2010



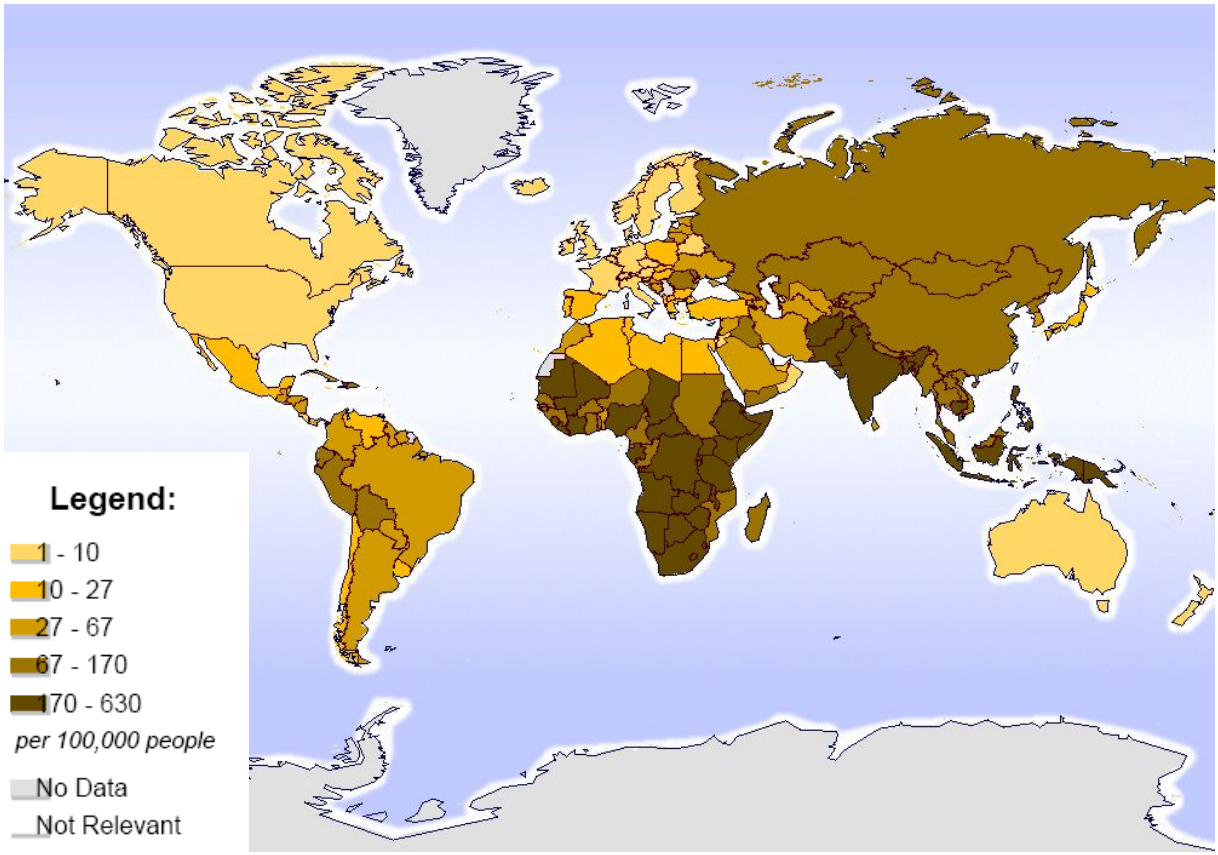
Mycobacterium tuberculosis (MTB) is the world's most deadly bacterial pathogen (4). About one-third of the world population is infected with MTB. By 2000 there were 8.3 million new cases and up to 2.2 million deaths per year due to tuberculosis (TB) (5). HIV infection is a potent risk factor for TB. The interaction of TB and HIV is formidable: rates of active TB are higher in HIV infected persons than in persons without HIV, because HIV increases the risk of reactivation of latent MTB (6) as well as the risk of rapid TB progression after infection or reinfection with MTB (7). HIV also increases TB transmission rates in the general community (8). Patients co-infected with TB/HIV exhibit higher rates of mortality than CD4-matched controls with TB but without HIV infection (9). The TB burden in countries with a generalized HIV epidemic has therefore increased rapidly over the past couple of decades, especially in the severely affected countries of eastern and southern Africa (2;10) **Figure 5**. In these countries up to 31% of new TB cases are attributed to HIV infection (5). In Tanzania, new TB cases increased five fold from 11,753 in 1983 to 61,603 in 2001 largely due to HIV/AIDS (11). TB therefore has become the most common disease associated with HIV/AIDS and the leading cause of morbidity and mortality (12).

Unlike the straightforward diagnosis and typical presentation of pulmonary tuberculosis (PTB) in HIV-1 seronegative individuals (13;14), PTB in HIV/AIDS frequently has atypical clinical and radiographic presentation (15-17). Often PTB is hardly distinguishable from other

causes of pulmonary infection on clinical and radiographic basis (18). This makes the diagnosis of TB in HIV infection considerably difficult, particularly in view of the resource limitations in the highly affected countries of sub-Saharan Africa. This amounts to delayed diagnosis and treatment, which affect the control strategies. To-date HIV infection is the single most potent factor for increase in TB morbidity and mortality in the African sub-continent.

Consequently, this deadliest TB/HIV tandem will result into approximately one billion new MTB infections in the world, over 150 million people with TB disease, and up to 36 million deaths due to TB between now and 2020 if the current trend is not abated (www.iuatld.org. 2002).

Figure 5. Tuberculosis cases per 100,000 people (2001)



Source: Globalis/UNEP/Global Virtual

This alarming magnitude of the TB disease asks for renewed and alternative control strategies directed mainly to the most affected regions "the TB/HIV Hot spots". Researches are needed specifically from these "Hot spots" to understand the pathophysiology behind TB disease in HIV co-infection. Relevant research questions are for instance; why do some individuals develop the disease while other don't?, what are the mechanisms leading to the atypical presentation of the disease?, what are the factors associated with high mortality?, what are the current MTB subtypes in the wake of high transmission rate due to HIV infection? We

equally need to take bold operational approaches by re-evaluating PTB diagnostic methods in HIV infection given the sputum scarcity associated with AIDS (19) and by monitoring the effectiveness of currently used antibiotics for TB treatment.

Current information available from sub-Saharan Africa on TB/HIV co-infection is insufficient: studies from this most affected part of the globe are sparse and not optimal. Only in few studies on TB in HIV infection from this region samples from the bronchoalveolar compartment (i.e. bronchoalveolar lavage fluid) have been used. This is important for better understanding the disease as the actual events take place in the broncholveolar parts of the lungs, and because of the sputum scarcity in AIDS patients (19). Accurate diagnosis of TB is important in understanding and characterizing the disease. Combination of microbiological and molecular techniques offers a more accurate diagnosis of TB and avoids erroneously assigning a patient as either TB positive or negative. However data on such an approach for diagnosing TB are lacking from this part of the world. Similarly, from this region no studies are available which characterize a broad range of cytokines and their interaction in TB/HIV co-infection. Such an approach is important given the immunological complexity associated with HIV infection.

For this reason we conducted pathophysiological and operational studies in an attempt to shed more light on the TB/HIV co-infection in a sub-

Saharan African country, using bronchoalveolar lavage fluid from accurately defined TB patients with HIV co-infection. All subjects studied in this work were indigenous Tanzanians, i.e. a true representation of one of the most affected countries of sub-Saharan Africa (10)

Outline of the thesis

Chapter 1.

In the introductory chapter the complex relationship between pulmonary tuberculosis (TB) and HIV infection is highlighted in the context of the HIV epidemic in tropical Africa and Tanzania in particular. Also, an outline is given of the research reported in the following chapters of this thesis.

Chapter 2.

In this study the ten-year trend in leading causes of hospitalisation and in-hospital morbidity and mortality attributed to HIV-infection is examined in a tertiary health care facility in northern Tanzania. Also, the prevalence of HIV infection in in-patients and the proportion of patients unaware of HIV infection as the cause of their ailing health are examined.

Chapter 3.

In this chapter the aetiological agents of pulmonary infection in a cohort of adult HIV infected patients are determined and correlated with the levels of immunosuppression, clinical presentation, chest radiographic findings and prognosis.

Chapter 4.

Due to an ongoing breakdown of cellular immunity in TB/HIV dual infection, the host is subject to a variety of infections including viruses. In this study we determine the prevalence of the most

common human herpes viruses in bronchoalveolar lavage (BAL) fluid and discuss their possible contribution to the high mortality in patients with TB/HIV co-infection.

Chapter 5.

Accurate microbiological diagnosis is crucial for TB control in the era of TB/HIV co-infection, particularly since clinical and radiographic features cannot adequately differentiate TB from other causes of pulmonary infection. In this study data are presented on the performance of diagnostic laboratory tests for pulmonary TB, including microbiological, serological and molecular methods (including real time PCR), using sputum, bronchoalveolar lavage fluid and serum in a TB and HIV endemic setting.

Chapter 6.

This chapter presents the results of a study into the dominant genotypes of *Mycobacterium tuberculosis* (MTB), determined by spoligotyping, in HIV infected and non-HIV patients in Tanzania. The genotypes are correlated with anti-TB drug sensitivity patterns. Also, the results of genotypic and phenotypic anti-TB drug susceptibility testing are compared.

Chapter 7.

In patients not infected with HIV, the role of macrophage migration inhibitory factor (MIF) in the course of MTB infection showed that MIF levels were determined by dose and virulence of the MTB strain and that high MIF levels were associated with fatal outcome. We report data on MIF in HIV infected patients with pulmonary TB or other pulmonary infections compared to TB patients without HIV infection and healthy controls. Also, the association of MIF levels with mortality is examined.

Chapter 8.

Our understanding of atypical presentation of tuberculosis in advanced AIDS, such as the lack of upper lobe cavitation, is insufficient. In particular it is not clear which events take place at the site of infection in the lungs. This study examines the local immune response in HIV infected patients with TB compared with HIV infected patients with non-TB pulmonary disease from Tanzania, using bronchoalveolar lavage (BAL) fluid. It correlates chest radiographic features and CD4 T cell level with levels of a panel of cytokines/chemokines present in BAL.

Chapter 9.

Infection with MTB is common to citizens of sub-Saharan Africa, however only a proportion of individuals develop active TB while in the majority the disease remains latent. Toll-like receptors (TLR) are important for host defence against MTB. TLR4 299 functional polymorphism is common in sub-Saharan Africa. Therefore, we investigated the association between TLR4 Asp 299Gly polymorphism and the development of active TB in HIV infected patients and examined whether there was an association with level of CD4 T cells.

Chapter 10.

This chapter discusses the findings from the above studies, puts the findings into perspective, and winds up by outlining avenues for future research.

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Chapter 2

HIV-associated morbidity, mortality, and diagnostic testing opportunities among inpatients at a referral hospital in Northern Tanzania

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ABSTRACT

Background

Hospitalized persons with HIV infection are among the most likely to benefit from expanding availability of antiretroviral therapy in sub-Saharan Africa.

Methods

Between 1990 and 2000, 3,667 HIV-infected persons were admitted to Kilimanjaro Christian Medical Centre in Moshi. Annual inpatient mortality rate ranged from 15-21%, and the proportion of female patients increased significantly during this time from 45 to 52 % ($p < 0.001$). Charts were abstracted from 1,683 HIV-infected patients admitted between 1996 and 2001.

Results

The most prevalent diagnoses in adults were pulmonary tuberculosis (21%), malaria (14%), and gastroenteritis/diarrhoea (12%), and those for children were non-tuberculosis pulmonary infection (21%), pulmonary tuberculosis (19%), and gastroenteritis/diarrhoea (12%). The relative risk of inpatient death was greatest for adults presenting with meningitis (RR 2.3, 95% CI 1.7-3.0), septicaemia (RR 2.0, 95% CI 1.2-3.3), and renal disease (RR 1.9, 95% CI 1.2-2.9), and mortality was higher for men than women (OR, 1.4, $p <0.005$). A single day point-prevalence survey in September 2001 identified HIV infection in 21% of those surveyed; 44% were not known to be infected.

Conclusion

HIV infection remains a major cause for admission and mortality in this population. A policy of routine testing would increase case finding and potentially enhance care and prevention efforts.

INTRODUCTION

Of the 42 million persons living with HIV/AIDS at the end of 2002, over 70 percent resided in Sub-Saharan Africa, making it easily the most affected region of the world (1). In Tanzania specifically, by the end of 2001, approximately 2.2 million individuals aged 15 and above were estimated to be living with HIV/AIDS, a 3% increase from the previous year. In the Kilimanjaro Region the prevalence of HIV infection among women attending some antenatal clinics has nearly tripled since 1992, with estimates between 1997 and 2000 ranging from 17 to 20% (2). Between 1992 and 1998, across 51 villages surveyed in Hai District of this region, HIV/AIDS accounted for 57% of deaths, reflecting the dramatic impact of this disease among younger, sexually active adults (3). Unfortunately, in Tanzania as in other countries in sub-Saharan Africa, many persons infected with HIV are not aware of their serostatus. This hampers efforts to prevent transmission and limits access to treatment and care services. A growing number of HIV voluntary counseling and testing (VCT) sites are beginning to address this problem. However, missed opportunities may occur when persons presenting to hospitals are not offered HIV testing.

As increased international attention and resources are focused on the AIDS crisis in sub-Saharan Africa and plans for more intensive and effective therapeutic interventions are developed, it has become increasingly important to describe the clinical manifestations of hospitalized patients (who likely have advancing disease), as this population could derive immediate benefit from antiretroviral medications. Others have reported hospital-based data from other regions in Tanzania (4) and elsewhere in sub-Saharan Africa (5-7), but to date, there have been no descriptions of hospital-based patient series of HIV infection in Northern

Zone of Tanzania. Recently, a policy of offering VCT to all persons admitted to hospitals in sub-Saharan Africa has been advocated (8). However, the number of additional HIV infections that might be detected with this method has not been estimated.

To describe clinical characteristics of HIV-infected persons and HIV seroprevalence among patients admitted to the major referral hospital in Northern Tanzania, we reviewed medical records of patients found to be HIV-positive over an 11-year period and offered widespread VCT testing in the context of a cross-sectional seroprevalence survey. To calculate the proportion of HIV infections missed by a testing policy based on clinical and behavioral criteria, we compared data from current testing strategy with those from the cross-sectional seroprevalence survey.

MATERIALS AND METHODS

Study site

Kilimanjaro Christian Medical Centre (KCMC) is located in Moshi municipality in the Kilimanjaro Region of Northern Tanzania. As one of four national referral centers, the 450-bed hospital serves >10 million persons living in the Northern and Central Zones of Tanzania. In addition, KCMC hosts a medical school and 15 other schools of allied health sciences. In 2001, 17,812 admissions and 1,121 inpatient deaths were recorded, and bed occupancy was nearly 100%.

Hospital inpatient series

Between 1990 and 2001, patients suspected by clinicians at KCMC to be at risk for HIV infection were routinely offered HIV testing; testing was not offered

routinely to all inpatients. According to World Health Organization guidelines (9), patients were tested for HIV antibody with two sequential rapid tests, commonly Capillus (Trinity Biotech, Wicklow, Ireland) and an enzyme immunoassay (Vironosticka HIV Uni-Form II Ag/Ab Microwell EIA, Biomerieux, France system). Demographic information and the status of the patient at discharge were extracted from discharge and HIV testing logs. An additional set of data, based on a chart review, was generated for patients admitted between 1997 and 2001 using a standardized form. These data included patient age, gender, status at discharge, and admission diagnoses. The recorded admission diagnoses reflected the opinions of senior consultant physicians who reviewed cases on the day of admission; if further investigation resulted in an alternate definitive diagnosis, this was recorded as the admitting diagnosis. For analysis, composite diagnostic categories were formulated by combining etiologically or syndromically related diagnoses to shorten the list of the numerous diagnoses observed in the data.

Cross-sectional seroprevalence survey

To assess the seroprevalence in this hospitalized population and, specifically, to help establish estimates of unsuspected HIV disease, a point prevalence survey was performed on September 18, 2001. The protocol was approved by the Ethical Clearance Committee at KCMC, and patients were enrolled only after informed consent was obtained. All patients on the general medical and pediatric wards at KCMC were approached to participate in the study. On the pediatric wards the child's guardian or parent provided consent. Patients who were younger than 6 months of age or were admitted to the intensive care unit or private medical service were excluded from the study. Clinical information,

including the patient's HIV serostatus known at the time, was abstracted from patient's charts and directed historical information and physical examinations targeting the World Health Organization surveillance case definition for AIDS (10) were performed on study participants. Blood was obtained by fingerstick and four drops of blood were transferred to filter paper, allowed to air dry, and sealed in plastic for transfer to Duke University Medical Center. A waiver for anonymized testing for HIV antibody was granted by the Investigational Review Board. Bloodspot samples were then eluted from filter paper and HIV testing was performed using the Vironostika HIV-1 Microelisa system (Organon Teknika Corp., Durham, NC) according to the manufacturer's instructions. Samples testing positive were confirmed with repeat testing using the same kit.

Statistical issues

Statistical computations were made using JMP 4.04 (SAS Institute, Cary, NC) and Epi Info 2002 (CDC, Atlanta, GA).

RESULTS

Hospital inpatient series

The number of patients known to be HIV seropositive increased approximately 2-fold from the first half of the decade to the second (Figure 1), and the male: female ratio shifted around 1997 to reflect a preponderance of females. The proportion of female patients known to be HIV seropositive increased significantly over these 11 years ($P < 0.001$, Chi-square test for trend), from consistently <50% between 1990-1996 (with a low of 38% in 1994) to consistently >50% since 1998. The annual in-hospital mortality for these patients generally increased, ranging between 14.6 to 21.2% during this time.

More detailed data available for 1997-2001 describes 1,553 adults known to be HIV-infected: 814 (52%) female and 739 (48%) male patients. The median (range) age for the adult population (>13 years) was 35 (13-92) years and the median (range) age for those who died was 36 (13-77) years. Female patients were significantly younger (median age 33, range 13-92 years) than male patients (median age 38, range 14-80, rank-sum $P < 0.001$). In-hospital mortality data were available for 1,549 patients. Of the 386 inpatient deaths, 28% of men and 178 (22%) of women died as inpatients (OR of death for men vs. women, 1.4, 95% CI 1.1 –1.8, p =0.004).

Table 1 lists the diagnostic categories assigned to 1,242 HIV-infected adults between 1997 and 2001 and the inpatient mortality rates among patients with these diagnoses. Most prevalent was pulmonary tuberculosis, which was seen in 21% of patients, followed by malaria (13.6%), gastroenteritis/diarrhea (12.2%), and non-tuberculosis pulmonary infection (10.1%). Taken together, pulmonary infections accounted for nearly one-third of all admissions. Whereas central nervous system disease was more frequently recorded in women than men (47 vs. 26, OR = 1.7, p =0.034), Kaposi's sarcoma (15 vs. 30, OR = 0.4, p = 0.009) and renal disease (9 vs. 18, OR = 0.5, p = 0.04) were seen more frequently in men. No significant gender differences were seen among other diagnoses. There was little variation in the median patient age across the diagnostic categories with the exception of intraabdominal infections (median age, 42; range, 24 to 57 years). Mortality rates were highest for those presenting with meningitis (RR 2.3, 95% CI 1.7– 3.0), septicaemia (RR 2.0, 95% CI 1.2 –3.3), renal disease (RR 1.9, 95% CI 1.2 –2.9) and non-tuberculosis pulmonary infections (RR 1.6, 95% CI 1.2 – 2.1).

Table 3 shows the diagnoses assigned to 130 paediatric patients between the ages of 2 and 13 years who tested positive for HIV-1 antibody. High prevalence rates of chest disease, particularly with non-tuberculosis pulmonary infection, were noted in this population. Whereas the median age for most diagnostic categories was quite young, that for extrapulmonary tuberculosis was 9 years (range 2-10 years).

Cross-sectional seroprevalence survey

On September 18, 2001, anonymous HIV testing was offered to all patients admitted to the general internal medicine and pediatric wards. Of the 61 adults on the medicine wards and 29 children on the pediatric wards offered testing, consent was provided by 58 adult patients (median age, 45; range 20-94 years) and by the guardians of 25 children (median age 1.9, range 0.7 to 14 years). Twelve adults (21%) and 4 of the 16 children over 18 months of age (25%) were found to be HIV-infected by antibody testing. Four (33%) of the adult patients were not known to be HIV seropositive. One of the four HIV-seropositive children was previously thought to be HIV-seronegative and two had unknown HIV-serostatus. No significant differences in sex or age were found between those testing HIV-antibody positive versus those who tested negative. Fifteen percent of the 26 women and 25% of the 32 men tested were HIV-infected ($p = 0.3686$), and the mean age of those infected versus uninfected was 44 years versus 52 ($p = 0.2415$). For the adult inpatients, the sensitivity of the WHO case definition for AIDS surveillance was 0.58 (95% CI, 0.31-0.74). For specific symptoms and signs the sensitivity was 0.75 (95% CI, 0.47-0.91) for weight loss, 0.33 (95% CI, 0.47-0.91) for chronic diarrhea, 0.50 (95% CI, 0.25-075) for prolonged fever, 0.58 (95% CI, 0-32-0.81) for cough, and 0.25 (95% CI, 0.09-0.53) for thrush.

DISCUSSION

We describe the diagnoses and mortality associated with HIV infection in a large referral hospital for the Northern Zone of Tanzania based on retrospective but systematic review of medical records. Our findings, consistent with those of others in sub-Saharan Africa, highlight the morbidity and mortality of HIV/AIDS seen in a relatively young, potentially economically productive population and the number of missed cases. We further describe increasing hospitalization rates for women over the decade of the 1990's, enumerate mortality rates associated with the most common hospital presentations among those known to be HIV-infected, and estimate the yield of a more aggressive testing strategy.

Significant gender differences in age and mortality were noted. The longitudinal data document an increasing proportion of women hospitalized with known HIV infection, and the women in our study were approximately 5 years younger than the men. Others have noted similar age differences in hospital-based studies in Kenya, Uganda, and Malawi (5-7). It remains unclear why hospitalized women are generally younger than men, but this may be explained by differences in age at acquisition of HIV (11), health seeking behaviour, and/or differences in the rate of progression. The hospital mortality rate for women was 22% whereas that for men was 28% ($p = 0.004$). Others have noticed more striking differences in gender-associated mortality in sub-Saharan Africa, speculating that cultural reasons may account for such differences; for example, very sick women may not be taken to the hospital as frequently as very sick men (5). The most prevalent diagnoses recorded in adults were pulmonary tuberculosis (21%), malaria (14%), gastroenteritis/diarrhoea (12%), and non-tuberculosis pulmonary infection (10%). Excepting malaria, the prevalence of these findings

are similar to findings in those reported from other regions of sub-Saharan Africa (5;7;12;13). Among patients known to be HIV-infected at KCMC, very high inpatient mortality was observed among patients with diagnoses of meningitis, septicaemia, renal disease, and non-tuberculosis pulmonary infection (inpatient death rates of 52, 47, 44, and 36%, respectively). Although relatively high death rates are expected with meningitis, septicaemia, and renal disease even among patients not infected with HIV in resource-poor settings, the relatively high death rate among patients with non-tuberculosis pneumonia is surprising and highlights the need for more intense investigations of chest disease in this population. We suspect that the case fatality rate for several of these diagnoses (e.g. cryptococcal meningitis) is higher than the inpatient mortality rate reported, as many of these patients were likely discharged home to die.

Our single-day point-prevalence survey documented HIV seroprevalence of 21% on the adult ward and 25% on the paediatrics ward. Within this small sample alone, the HIV serostatus was not previously known in 44% of HIV-infected patients, reflecting a sizeable proportion of patients who are unaware that they are infected. The greatest limitation to this serosurvey was the small sample size. A prevalence study conducted over a period of a few weeks may give a more precise picture of the prevalence of HIV seropositivity and clinical symptomatology at KCMC. Despite these limitations, our data suggest that using a testing policy directed by clinical suspicion, a considerable number of HIV-infections go undiagnosed. De Cock et al. have argued for routine testing of all those admitted to general medical wards (8). This 'opt-out' policy of offering HIV testing to all persons admitted irrespective of perceived risk would detect

many more patients living with HIV/AIDS and, therefore, would provide additional prevention and treatment opportunities.

The relatively low seroprevalence at KCMC compared with other similar studies in sub-Saharan Africa (4-6;12;14) may reflect regional differences in overall seroprevalence. An alternative explanation is the substantial burden of chronic HIV/AIDS care taken on by the community, including the regional activities of a well-organized home-based care program by KIWAKKUKI which cares for between 700 and 1000 patients in the surrounding Kilimanjaro Region (personal communication, Lightness Kaale). Notably, at Kenyatta National Hospital in Nairobi, despite predictions of overwhelming numbers of AIDS cases, a decrease in clinical AIDS presentations was noted between 1992 and 1997 (5).

Although HIV seroprevalence is not as high in the Kilimanjaro Region as in many other parts of sub-Saharan Africa, it remains a significant contributor to the inpatient census and mortality in this referral hospital. HIV infection has increasingly affected women, and disproportionately affects an otherwise economically productive segment of the population. In our relatively small point-prevalence survey, over 40% of patients in this facility who were HIV-infected were not known to be so. A more liberal testing strategy would likely identify additional individuals, giving them opportunity to access expanding HIV care options, including antiretroviral therapy, and to receive targeted prevention education to prevent further transmission.

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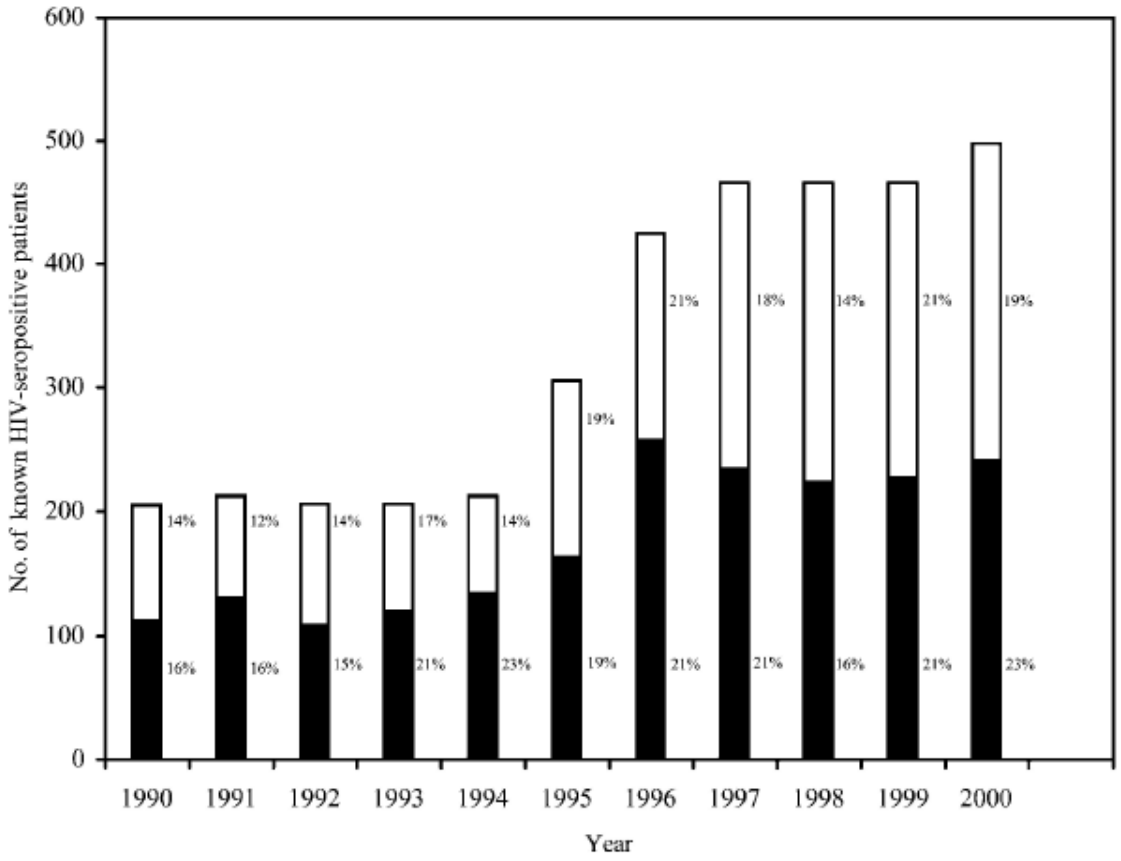

FIG. The annual numbers of male (■) and female (□) inpatients at the Kilimanjaro Christian Medical Centre who were known to be infected with HIV. The percentages shown next to the bars indicate the corresponding levels of inpatient mortality.

TABLE 1. *Diagnoses assigned to 1242 HIV-infected adult inpatients at the Kilimanjaro Christian Medical Centre between 1997 and 2001*

Diagnostic category*	*N*	Prevalence (%)	Female:male ratio	Median age (years)	No. of inpatient deaths	Mortality (%)	Crude odds ratio and (95% confidence interval) for inpatient mortality†
Pulmonary tuberculosis	262	21	1.0	37	62	24	1.0 (0.7–1.4)
Malaria	169	14	1.1	34	52	31	1.5 (1.1–2.2)
Gastro-enteritis/diarrhoea	152	12	1.1	34	31	20	0.8 (0.5–1.2)
Non-tubercular pulmonary infection	126	10	0.9	36	45	36	1.9 (1.3–2.8)
Oral candidiasis	114	9	1.4	35	22	19	0.7 (0.5–1.2)
Anaemia	73	6	1.1	36	25	34	1.7 (1.0–2.9)
Central-nervous-system disease	73	6	1.8	37	20	27	1.2 (0.7–2.1)
Dermatitis	48	4	1.1	37	1	2	0.1 (0.01–0.4)
Meningitis	46	4	1.0	34	24	52	3.7 (2.1–6.7)
Kaposi's sarcoma	45	4	0.5	33	6	13	0.5 (0.2–1.1)
Extrapulmonary tuberculosis	43	4	1.2	35	9	21	0.8 (0.4–1.8)
Cardiac disease	34	3	1.1	35	10	29	1.3 (0.6–2.8)
Renal disease	27	2	0.5	35	12	44	2.6 (1.2–5.7)
Malignancy‡	27	2	2.0	37	5	19	0.7 (0.3–1.9)
Urinary-tract infection	24	2	1.7	34	4	17	0.6 (0.2–1.9)
Lymphoma	23	2	0.6	38	5	22	0.9 (0.3–2.4)
Septicaemia	19	2	1.4	33	9	47	2.9 (1.2–7.3)
Skin/soft-tissue infection	18	1	0.6	35	1	6	0.2 (0.02–1.4)
Sexually transmitted disease	18	1	2.6	36	5	28	1.2 (0.4–3.5)
Intra-abdominal infection	16	1	1.3	42	0	0	–
Cryptococcosis	13	1	1.2	35	3	23	1.0 (0.3–3.5)
Otitis media	8	1	1.7	36	3	38	1.9 (0.5–8.1)
Other, unclassified diagnoses	142	11	1.2	35	18	13	0.6 (0.4–1.0)

*Multiple diagnoses per patient were possible; the mean number of diagnoses/patient was 1.2 (with a range of one to three).
†Calculated with death as the dependent variable and diagnostic category as the independent variable.
‡Other than Kaposi's sarcoma or lymphoma.

TABLE 2. *Diagnoses assigned to 130 HIV-infected paediatric inpatients (aged 1.5–12 years) at the Kilimanjaro Christian Medical Centre between 1997 and 2001*

Diagnostic category*	*N*	Prevalence (%)	Median age and (range) (years)	No. of inpatient deaths	Crude odds ratio and (95% confidence interval) for inpatient mortality†
Non-tubercular pulmonary infection	27	21	4 (1.5–10)	8	2.3 (0.8–6.1)
Pulmonary tuberculosis	25	19	4 (2–12)	3	0.5 (0.1–2.0)
Gastro-enteritis/diarrhoea	16	12	3 (1.5–10)	6	3.2 (1.0–9.9)
Malnutrition	15	11	3 (2–9)	6	3.6 (1.1–11.3)
Malaria	14	11	3.5 (2–11)	3	1.2 (0.3–4.8)
Otitis media	6	5	4 (2–12)	0	–
Anaemia	7	5	4 (2–9)	2	1.8 (0.3–10.1)
Extrapulmonary tuberculosis	10	8	9 (2–10)	0	2.0 (0.5–8.5)
Septicaemia	7	5	3 (2–9)	1	3.6 (0.8–17.4)
Kaposi's sarcoma	6	5	3.5 (2–8)	1	0.9 (0.1–7.8)
Other	16	19	4 (1.5–12)	1	0.3 (0.03–2.1)

*Multiple diagnoses per patient were possible.
†Calculated with death as the dependent variable and diagnostic category as the independent variable.

Chapter 3

Aetiology and presentation of AIDS–associated pulmonary infections in a referral hospital in northern Tanzania

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ABSTRACT

Background

The objectives of this study were to determine the aetiological agents of pulmonary infections in HIV-infected Tanzanians and to correlate the causative agents with clinical, radiographic features, and one-month mortality.

Methods

In a prospective study at KCMC hospital we obtained bronchoalveolar lavage fluid (BAL) from 120 HIV infected patients with pulmonary infections. We analyzed BAL for causative agents.

Results

Causative agents were identified in 71 patients (59.2%) and multiple agents were in 16 patients. Common bacteria were identified in 35 patients (29.2%), *Mycobacterium tuberculosis* (MTB) in 28 (23.3%), *Human Herpes Virus 8* (HHV8) in 12 (10%), *Pneumocystis jiroveci* in 9 (7.5%) and fungi in 5 (4.2%) patients. Median CD4 T cell count of the patients with identified causes was 47 cells/μl (IQR 14-91) and in the 49 patients with undetermined aetiology was 100 cells/μl (IQR 36-188; p=0.01). Micronodular chest radiographic lesions were associated with presence of MTB (p=0.002). The one-month mortality was 20 (16.7%). The highest mortality was associated with HHV8 (41.7%) and MTB (32.1%). Mortality in patients with undetermined aetiology was 11.3%. No death occurred in patients with PCP.

Conclusion

In this study of severely immunosuppressed HIV-infected patients with pulmonary infection a variety of causative agents was identified. Micronodular radiographic lesions were indicative of TB. High mortality was associated with *M. tuberculosis* or HHV8. No death occurred patients with *P. jiroveci* infection.

INTRODUCTION

Sub-Saharan Africa is the region of the world most affected by HIV/AIDS, more than 70% of HIV infected individuals live in this African sub-continent, (1) and the HIV pandemic is still increasing. Despite this, data pertaining to the disease presentation from this region compared to the developed world are scarce. The clinical presentation of HIV/AIDS and the occurrence of opportunistic infections depend on different factors such as the presence of endemic diseases, quality of health services, availability of and access to anti-retroviral treatment, and levels of education of the population. Pulmonary infections are the leading causes of morbidity and mortality in HIV-infected individuals (2;3). A microbiological diagnosis is of crucial importance in HIV infected patients with pulmonary infections because of atypical clinical and radiographic manifestations and high mortality (4), Because in these settings resources are limited, treatment is largely empirical, but to be effective, it should be tailored to locally prevailing causative organisms. Therefore health facilities equipped with advanced diagnostic techniques should monitor the trend of diseases and obtain data on aetiological pathogens, so that empirical treatment regimens can be improved.

The aim of this study was to find the aetiological pathogens of pulmonary infections in HIV infected patients presenting with pulmonary infection. Furthermore, we correlated the pathogens identified with clinical and radiographic presentations, level of immunity, and outcome after one month.

MATERIALS AND METHODS

At Kilimanjaro Christian Medical Centre (KCMC) in northern Tanzania we enrolled 120 HIV infected patients of 18 years and above, who presented with features of

chest infection such as cough (dry or productive) and/or chest pain, dyspnoea, fever and chest radiographic abnormalities. These patients were referred by clinicians for bronchoscopy as part of patient's management. Pregnant women and patients with oxygen saturation less than 90% under 6 l/min of oxygen were excluded from the study.

Bronchoscopy and bronchoalveolar lavage (BAL) was performed by standard procedure using flexible fiberoptic bronchoscope. Briefly, the bronchoscope was wedged in one of the heavily involved segmental bronchi as seen on the chest radiograph. In case of diffuse lung involvement, the scope was wedged in one of the segmental bronchi of the right middle lobe. Then aliquots of 50 ml or less of sterile saline at body temperature, up to a maximum of 150 ml, were instilled and at least 40 ml was sucked back into a sterile container. The sediments of BAL fluid obtained by centrifugation at 1500 g for 10 minutes were used for laboratory tests to identify the aetiological agent of the chest infection.

For identification of *M. tuberculosis,* BAL samples were decontaminated with 4% NaOH, centrifuged and cultured using in-house made Lowenstein-Jensen (LJ) solid medium, with a maximum of 8 weeks incubation. Diagnosis of *M. tuberculosis* was based on positive culture results showing acid fast bacilli (AFB) in ZN stain.

Diagnosis of Pneumocystis pneumonia (PCP) was based on identification of *Pneumocystis (P) jiroveci* with at least one of the following techniques: Giemsa stain, Gomori methenamine silver (GMS) stain or immunofluorescence test (IFT). Also real time PCR was performed for detection of *P. jiroveci* DNA in the BAL fluid, using 40 as the cycle threshold (C_T) cut off value (5).

Conventional PCR was used for the diagnosis of *Human herpes virus 8* (HHV8) as described by Chang and collaborators (6).

Fungi were diagnosed by direct smear and culture for seven days using Sabouraud dextrose agar and for 21 days in Sabouraud dextrose liquid medium (Oxoid, Basingstoke, Hampshire, England). Cryptococci were identified microscopically in Giemsa stained slides and confirmed by Mucicarmine stain. Murex cryptococcal antigen test in serum was done as described by Jaye *et al* (7).

Significant growth of common bacteria was determined by routine culture procedures. Antibacterial susceptibility testing could not be done.

Peripheral blood samples were taken for total and differential white blood cell count (WBC), haemoglobin, HIV test and CD4 T cell count. Blood was tested for anti-HIV antibodies by both Capillus ™ HIV-1/HIV-2 rapid test (Trinity Biotech, Bray, Ireland), and Vironostika ® HIV uniform II ag/ab microwell enzyme immunoassay (bioMerieux, Marcy 1'Etoile, France). CD4 T cell counting was done by flow-cytometry technique (Becton Dickinson Facs Count machine with BD Facscount^{TM} reagent).

Chest radiographs were read by an experienced radiologist, who was informed that the patient had symptoms and signs of chest infection and were HIV infected, but who was not given other clinical information. Reading was done in all cases in a systematic way, describing for each chest film presence or absence of the following characteristics: nodules (miliary: < 3 mm; micronodules: 3-6 mm; macronodules: 6 mm – 3 cm); infiltrates (alveolar, interstitial or mixed), cavities and their diameter, and other abnormalities (pleural effusion or thickening, masses (> 3 cm), hilar and/or mediastinal adenopathy).

Data on outcome of the patients were obtained at the time of discharge and for outpatients after 4 weeks of follow up. All patients were given appropriate treatment based on the diagnosis reached by the attending physicians. The study in no way interfered with the management of patients. Results obtained from this study during the course of patient's management were used in the management as needed.

Data analysis was done by SPSS version 12-software for Windows. Normally distributed values were presented as mean with standard deviation (SD). In other cases data were expressed as median with interquartile range (IQR). Chi-square test was used to quantify correlations between dichotomous variables. Mann-Whitney U test and t-test were used to compare medians and means, respectively. P-value equal or less than 0.05 was regarded as statistically significant.

Clearance for the study was given by the Institutional (KCMC) and National (Tanzania) Ethical Review Boards and informed written consent was obtained from each patient or a close relative.

RESULTS

General characteristics of the study population

A total of 120 patients were enrolled. The mean age of the study population was 39 years (SD +/- 10), 54% was male. Median CD4 T cell count was 65 cells/µl (IQR 20 – 147). As shown in **Table 1**, most patients presented with cough, chest pain and difficulty in breathing. Of the 120 patients, 19 (15.8%) presented with signs and symptoms directly related to respiratory disease only, while the

remaining 101 (84.2%) had co-morbidities as well, such as oral candidiasis, different dermatological lesions, lymphadenopathy, wasting or diarrhea, **Table 1**. Of the 120 patients, 100 (83.3%) used empirical antimicrobial treatment prior to or at the time of enrolment in the study and 44 (36.7%) were on multiple antimicrobial agents. The most commonly used antimicrobial agents were trimethoprin-sulfamethoxazole (TMP-SMX), penicillins and fluconazole, used by 59 (49.2%), 52 (43.3%) and 38 (31.7%) patients, respectively. Some other antimicrobial agents used were: anti-mycobacterial agents 15 (12.5%) patients, and metronidazole, cephalosporins or chloramphenicol in 3-5 patients each.

Aetiology of pulmonary infection

In 71 (59.2%) of the 120 patients causative agents were identified. In the remaining 49 (40.8%) patients no organisms were found. In the 71 patients, 89 causative pathogens were isolated, in 14 two agents and in two three agents. In 35 (29.2%) patients common bacteria were isolated (*Streptococcus pneumoniae in 14, S. pyogenes in one*, *Staphylococcus aureus* in 7, coliform bacteria in 5, klebsiella species in 5 and *Pseudomonas aeruginosa* in 3) , in 28 (23.3%) *M. tuberculosis*, in 12 (10%) HHV8, in 9 (7.5%) *P. jiroveci* (identified microscopically) and in 5 (4.2%) fungi, (**Table 2**). The fungi identified were *Cryptococcus neoformans* in 3 patients and *Aspergillus fumigatus* in two. The cryptococcal cases were identified by a positive cryptococcal antigen test in serum (which was negative in the remaining 117 patients) and in one of the three also cryptococci were seen microscopically in the BAL fluid. In a few patients yeasts (candida, other) were seen in low concentrations, but these were regarded as contaminants from the oropharynx.

Real time PCR for *P. jiroveci* was positive in 17 patients, including the 9 cases in which *P. jiroveci* was seen microscopically. Of the 8 cases positive by real time PCR only, in 4 no other organisms were isolated and 2 of the eight were on treatment with TMP-SMX for clinical suspicion of PCP. The mean (SD) C_T value for *P. jiroveci* detected by both real time PCR and microscopic examination of stained BAL fluid was 27 (+/- 4) and the mean C_T value for *P. jiroveci* detected by real time PCR only was 35 (+/- 4), t – test = -20.4, p = 0.003. Only the 9 (7.5%) cases diagnosed microscopically as well were regarded as indicating PCP, thus explaining the pulmonary disease of these patients.

Among the 28 patients in whom *M. tuberculosis* was isolated, in 21 this was the only pathogen, while 7 cases had co-infection with common bacteria or (in one case) HHV8. In 7 cases *P. jiroveci* was the only pathogen isolated, while two patients had both *P. jiroveci* and coliform bacteria in BAL. Of the 35 patients in whom common bacterial pathogens were isolated, 18 had a single bacterial pathogen, the remaining had multiple types of infections. No cases of *M. tuberculosis* and *P. jiroveci* co-infection were found.

Correlation of aetiological agents with radiological and clinical parameters

Ten patients (8.3%) had normal chest radiographs. In six of these 10 patients microorganisms were isolated in BAL fluid; 1 *M. tuberculosis*, 4 common bacteria, and 1 *Aspergillus fumigatus* infection. The bacteria isolated included *Staphylococcus aureus*, *Streptococcus pneumoniae*, klebsiella and coliform bacteria, either single or in combination. In the remaining four patients with normal chest radiograph no organisms were isolated.

In 23 of the 28 TB patients chest radiograph showed nodular lesions, **Table 3**. Association between microbiological diagnosis of TB with the radiographic presence of nodules was statistically significant, $\chi^2 = 7.3$, $p = 0.008$. The diagnosis of TB was further statistically significantly associated with presence of the nodular subgroup of micronodules: 21 patients with TB had micronodules on the chest radiographs, $\chi^2 = 9.9$, $p = 0.002$. No significant association was found between TB and other types of radiographic features or between radiographic features and other identified pathogens.

Of 28 TB patients, 23 had low haemoglobin, $\chi^2 = 9.0$, $p = 0.005$. PCP was associated with dyspnoea; all 9 patients with PCP had dyspnoea, $\chi^2 = 8.1$, $p = 0.004$.

Seventeen patients (14.2%) had clinical Kaposi's sarcoma (KS) lesions **Table 1**; 10 on the skin, 5 on the palate, and 2 in the airway. Three patients had both detectable KS lesion and HHV8 DNA in the BAL fluid, two patients with lesions in the airway and the other one on the skin. No statistically significant association was found between presence of HHV8 in the BAL fluid and presence of detectable KS lesion.

Correlation between causative agents of the chest infection and level of immunity

The overall median CD4 T cell count for all patients in whom causative pathogens were found was 47 cells/ml (IQR; 14 – 91) while in patients in whom no organisms were found was 100 cell/ml (IQR; 36 – 188), (Mann-Whitney *U* test, p = 0.01). Patients with *M. tuberculosis* had median CD4 T cell count of 54 cells/ µl (IQR, 29 – 128), patients with HHV8 47 (IQR, 9 – 185), patients with common bacterial pathogens 44 (IQR, 10 -136), with *P. jiroveci* 26 (IQR, 7 – 91)

and patients with fungi 14 cells/ µl (IQR, 1 – 69). No statistically significant difference in CD4 T cell count was found between these groups.

Outcome

Twenty out of the 120 patients (16.7%) died during the one month follow up period. Nine of the 28 patients (32.1%) with TB died, in six of the nine patients *M. tuberculosis* was the only pathogen isolated, while of the other three, two also had co-infection with HHV8 and one with *Staphylococcus aureus*. Five of the 12 patients (41.7%) with HHV8 died; in two of the five patients HHV8 was the only pathogen isolated, while of the remaining three, two had co-infection with TB and one with *Streptococcus pneumoniae*. One of the three patients with *Cryptococcus neoformans* died; in this patient also *Staphylococcus aureus* and klebsiella bacteria were found. One patient died in whom *Staphylococcus aureus* was identified as the only pathogen. Six (12.2%) of the 49 patients with undetermined aetiology of chest infection died. None of the 9 patients with PCP died. *M. tuberculosis* and HHV8 were statistically significantly associated with mortality, $\chi^2 = 5.7$, $p = 0.02$ and $\chi^2 = 6.0$, $p = 0.03$, respectively.

DISCUSSION

In the studied population of severely immunosuppressed HIV-infected patients with pulmonary infection, the spectrum of pathogens identified was wide, ranging from common bacteria, *M. tuberculosis*, *P. jiroveci*, HHV8 and fungi. Common bacterial infection was the leading cause of pulmonary infection, however by far the most deadly single causative bacterial pathogen for chest infection was *M. tuberculosis*. One-third of the TB patients in this study died within one-month of follow up. The TB burden in countries highly affected by HIV

has increased tremendously and has generally resulted into high mortality (2;8-11). PTB/HIV co-infected patients exhibit a higher risk of death than other co-infections in HIV infected patients even when they are matched for CD4 T cell count, which is largely due to adverse interaction of the two infections (12;13). We found that presence of micronodules on chest radiograph, as well as anaemia, were statistically significantly associated with TB. Generally, features differentiating TB from other common pulmonary infections in HIV infected individuals are few (4;14;15). Given the sputum scarcity in HIV infection and low yield of acid fast bacilli (AFB) smear (16), presence of micronodular lesions in chest radiographs in the absence of other aetiological agents of respiratory infection in these patients should be highly suggestive of TB.

HHV8 was among the most common pathogens found in BAL in this study and presence of HHV8 was significantly associated with mortality. HHV8 DNA in BAL is mainly associated with Kaposi's sarcoma (KS) of the lungs with high sensitivity and specificity than cutaneous KS (17). Lung parenchyma is the primary site of pulmonary KS (18) and lesions are therefore easily missed by bronchoscopy. Pulmonary KS, unlike cutaneous KS, contributes decisively to morbidity and mortality especially in resource-poor setting where specific treatment is lacking (19). To our knowledge, we are the first to report the presence of HHV8 in BAL fluid from HIV seropositive patients from Africa. HHV8 is highly prevalent in sub-Saharan Africa and some parts of southern Europe but less common elsewhere (20). It can therefore be expected that Kaposi’s sarcoma also contributes significantly to pulmonary pathology in sub-Saharan Africa. Our findings are in line with this. Besides that, HHV8 has also been associated with primary effusion

lymphoma and multicentric Castleman's disease (21;22), which have very poor prognosis and are associated with HIV infection (23;24).

Prevalence of *P. jiroveci* in this study was higher than previously reported from Tanzania (25;26). However, this prevalence may still be an underestimation since patients with hypoxaemia despite administration of oxygen could not undergo bronchoscopy and thus were excluded from the study. Also, nearly 50% of patients had used TMP-SMX prior to enrolment into the study. Lower prevalence of PCP in Africa has been largely attributed to diagnostic problems, the current trend however shows an overall increase in PCP in developing countries (27;28) which could be due to increased awareness and improvement in the diagnosis of *P. jiroveci*. Additional use of real time PCR in this study showed a much higher prevalence of PCP than using standard staining techniques alone. This is due to the higher sensitivity of real time PCR compared to staining techniques (29). However, the median C_T value of the cases detected by real time PCR alone was significantly higher than that of cases detected by staining techniques as well as real time PCR. There was also no overlapping of the standard deviations of the C_T values. This finding suggests that the validity of this rapid molecular technique to distinguish between clinical PCP disease and colonization lies on the application of an appropriate C_T cut off value (29). High prevalence of PCP in this study was not associated with mortality since none of the patients with PCP died during the one-month follow up. This may be due to our clinical practice of applying early presumptive treatment with TMP-SMX when PCP is suspected. This implies that in this way the high mortality associated with PCP is preventable especially in patients presenting with dyspnoea, a symptom which was found to be significantly associated with

detection of *P. jiroveci* in BAL fluid. Instituting presumptive treatment has been found to lower mortality in patients with PCP (30).

Common bacteria were also highly prevalent in this study, despite the fact that the majority of patients used empirical antibiotic treatment prior to enrolment into the study. A similar trend has been observed in other studies in Africa with streptococcal pneumonia being the commonest (3;15;26;31-33). Usually common bacteria occur in patients with relatively preserved CD4 T cell count, but a recent study in north Africa also found that common bacteria were more likely to occur at lower CD4 T cell count, (3) and thus common bacteria should also be suspected even in advanced HIV/AIDS.
Few fungal infections (three *Cryptococus neoformans*, two *Aspergillus fumigatus*) were identified in this study. The incubation period of twenty-one days used for fungus culture, however, may have hindered identification of *Histoplasma capsulatum*. In the two patients in whom only cryptococcal antigen test was positive, cryptococcal pulmonary infection was entertained as the cause of morbidity since no other manifestations of cryptococcal infection (such as meningitis) were found.

Other serious co-morbidities not pertaining to pulmonary infection were also quite prevalent in this study. These conditions which usually confound morbidity are quite common in HIV infected patients from this region (15;26).

In more than 40% of patients no causative organisms were identified. Part of this may be attributed to the fact that over 80% used empirical antimicrobial treatment prior to the laboratory investigations, but we may have missed

pathogens undetectable by our diagnostic methods. This group of patients had a somewhat higher median CD4 T cell count and a significantly lower mortality rate than the patients in whom causative agents were isolated. In other studies the percentage of patients with undetermined aetiology in HIV infection varies, but may be almost half of the study population (15;33).

In conclusion, in this group of severely immunosuppressed patients with pulmonary infection, we found a wide spectrum of aetiological agents. The main identified pathogens were *M. tuberculosis, Streptococcus pneumoniae / S. pyogenes*, HHV8, *P. jiroveci* and *Staphylococcus aureus.* Mortality was high, particularly in TB patients and in patients with HHV8. Micronodular chest radiographic lesions and anaemia were associated with TB. PCP was significantly associated with dyspnoea.

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Table 1. Clinical and laboratory features of the study population (n=120)

Symptoms	
Cough, n (%)	118 (98.3)
Chest pain, n (%)	99 (82.5)
Dyspnoea, n (%)	65 (54.2)
Wasting, n (%)	37 (30.8)
Diarrhea, n (%)	15 (12.5)
Use of antibiotics prior to admission, n (%)	100 (83.3)
Signs	
Oral candidiasis, n (%)	50 (41.7)
Fever, n (%)	41 (34.2)
Skin lesions* n (%)	38 (31.7)
Lymphadenopathy, n (%)	19 (15.8)
Kaposi's sarcoma lesion, n (%)	17 (14.2)
Herpes zoster lesion, n (%)	16 (13.3)
Herpes simplex, n (%)	3 (2.5)
Respiratory rate, mean (SD)	27 (9)
Laboratory tests	
Oxygen saturation in %, mean (SD)	92 (8)
CD4 T cell count (cells/μl), median (IQR)	65.0 (20 – 147)
Haemoglobin (g/l), median (IQR)	90.0 (77 – 115)
WBC count (x 10^9/l), median (IQR)	5.5 (3.7 – 8.4)
Neutrophil count (x 10^9/l), median (IQR)	2.5 (1.6 – 3.0)
Lymphocyte count (x 10^9/l), median (IQR)	1.6 (1.1 – 3.0)
ESR (mm/hr), median (IQR)	70 (117 – 136)

- Other than Kaposi's sarcoma and herpes zoster lesions

Table 2. *Causative agents in 120 HIV-infected patients with pulmonary infection*

AGENTS *(*)*	FREQUENCY	%
M. tuberculosis	28	23.3
S. pneumoniae / S. pyogenes (**)	15	12.5
Human herpes virus 8	12	10.0
Pneumocystis jiroveci	9	7.5
Staphylococcus aureus	7	5.8
Coliform bacteria	5	4.2
Klebsiella species	5	4.2
Cryptococcus neoformans	3	2.5
Pseudomonas aeroginosa	3	2.5
Aspergillus fumigatus	2	1.7
No organism isolated	49	40.8

(*) More than one causative agents were found in 16 patients: 14 patients with two causative agents, and two patients with three.
(**) *Streptococcus pneumoniae* 14; S*treptoccus pyogenes* 1.

Table 3. *Radiographic features of different causative pathogens (n, %)*

PATHOGEN	MILIARY	NODULES	INFILTRATES	CAVITIES	HILAR NODES	PLEURAL EFFUSION	NORMAL
MTB (n = 28)	1 (3.6)	23(82.1)	13(46.4)	7 (25)	6 (21.4)	6 (21.4)	1 (3.6)
Bacteria (n = 35)	2 (5.7)	15 (44.1.)	14 (40.0)	3 (8.8)	2 (5.9)	4 (11.7)	4 (11.7)
HHV8 (n = 12)	1 (8.3)	5 (41.7)	7 (58.3)	1 (8.3)	2 (16.8)	5 (41.7)	0 (0)
P. jiroveci (n = 9)	0 (0)	5 (55.6)	5 (55.6)	0 (0)	0 (0)	1 (11.1)	0 (0)
Fungi (n = 5)	1 (20)	2 (40)	3 (60)	0 (0)	1 (20)	1 (20)	1 (20)
No M.O*. (n = 49)	2 (5.7)	30 (61.2)	30 (61.2)	13 (26.5)	5 (10.2)	10 (20.4)	4 (2.0)

* No MO: no microorganism isolated

MTB: *Mycobacterium tuberculosis*

HHV8*: Human herpes virus 8*

Chapter 4

Human Herpes Viruses in bronchoalveolar lavage fluid of HIV infected Tanzanians with Tuberculosis or other lung disease

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ABSTRACT

Background

During the first month of treatment of pulmonary tuberculosis (TB), mortality among TB/HIV co-infected patients is high and the cause is unclear. Human herpes-viruses (HHV) are common in immunosuppression. The interaction between TB and HHV is unclear.

Methods

We determined by PCR the prevalence of *Epstein-Barr virus* (EBV), *Cytomegalovirus* (CMV), *Herpes simplex viruses* 1 and 2 (HSV-1 and HSV-2) and *Human Herpes Virus 8* (HHV8) in bronchoalveolar lavage (BAL) fluid of 50 HIV infected Tanzanian patients with evidence of chest infection: 25 with TB and 25 with other chest infections. We correlated the one-month mortality in both groups to the HHV prevalence.

Results

In 41 (82%) patients, HHV were identified; 21 in the TB and 20 in the non-TB group. Prevalence of EBV (78%) and CMV (24%) were the highest. Ten patients (20%) died within one-month, 8 (32%) TB and 2 (8%) non-TB patients (p=0.11). HHV was not an independent predictor of fatal outcome.

Conclusion

In this study we found a very high prevalence of human herpes viruses in the BAL fluid of symptomatic and severely immunosuppressed HIV-infected Tanzanian patients. Prevalence of HHV was not associated with presence of TB, and not associated with mortality in TB patients in one-month follow up.

INTRODUCTION

Pulmonary tuberculosis (TB) is commonly associated with Human Immunodeficiency (HIV) infection, especially in the severely HIV-affected countries of eastern and southern Africa, like Tanzania where TB has increased rapidly in the past decades.(1;2) Despite low levels of resistance of *M. tuberculosis* (MTB) to anti-tuberculosis drugs,(3) about 30% of TB / HIV dually affected patients die during the course of treatment,(2-4) and more than half of the deaths occur within the first month of treatment. (2;4) The reasons for the high mortality are not well understood in the majority of the cases. Infection with the human herpes viruses (HHV) is ubiquitous. In HIV/AIDS, infection with *Cytomegalovirus* (CMV) or *Herpes simplex virus* (HSV) can result in pneumonia, and infection with *Human herpes virus 8* (HHV8) in Kaposi sarcoma of the lung.(5) HHV are frequently present in the bronchoalveolar lavage (BAL) fluid from severely immunocompromised HIV-infected patients with pulmonary symptoms, but not present in BAL fluid of healthy, immunocompetent individuals.(6)

In daily practice, especially in resource-limited settings which are highly burdened by both HIV and tuberculosis, viral infections are not routinely investigated in patients with respiratory symptoms. We therefore do not know the prevalence of HHV in TB/HIV co-infection, the association between HHV and TB in HIV and the role of these viruses in the outcome of patients with TB/HIV co-infection. Clinical studies have suggested that beta-herpes virus infection may increase the risk for other infections, the severity of infection, or the tempo of disease progression. For example, increased incidences of bacterial and fungal infections have been reported in association with CMV.(7) The cause of the

adverse effects of HHV may be due to the immunomodulatory effects of human beta-herpesviruses that have been reported in vitro and in vivo, as well as the interaction of these viruses with HIV-1.(7)

In this study among HIV infected patients with evidence of chest infection in Tanzania, we used PCR to determine the prevalence of the most common HHV: *Epstein-Barr virus* (EBV), CMV, HHV8 and HSV-1 and HSV-2 in BAL fluid. We determined the prevalence of HHV in patients with TB and those with non-tuberculous pulmonary infection (non-TB). Furthermore, we related the one-month mortality in both groups to the prevalence of HHV-DNA in BAL fluid.

MATERIALS AND METHODS

The study was performed at Kilimanjaro Christian Medical Centre (KCMC), Moshi, the reference hospital for the northern zone of Tanzania, between November 2003 and September 2004. We enrolled 50 HIV-infected patients with pulmonary infection, 25 with proven TB and 25 with non-TB pulmonary infections. The TB group was MTB positive by both Lowenstein-Jensen (LJ) culture and real time (rt) PCR and the non-TB group was MTB negative by both LJ culture and rtPCR of the BAL fluid. Culture was done by in-house made LJ, and rtPCR was done as described by Savelkoul and colleagues.(8) TB patients were randomly selected from a larger study on causes of chest infection in HIV infected patients with clinical evidence of chest infection and an abnormal chest radiograph. The matched control for each TB patient was identified as the first patient with non-TB lung disease appearing in the dataset who was of the same age (+/- 5 years) and comparable CD4 T cell count (+/- 25 cells/mm3).

To obtain BAL, patients underwent bronchoscopy with BAL using flexible fiberoptic bronchoscope (Olympus p45 Tokyo, Japan). Briefly , after routine

preparation of the patient, the bronchoscope was wedged in one of the heavily involved segmental bronchi as seen on the chest radiograph, or in the middle lobe in case of diffuse lung involvement. Then aliquots of 50 ml of sterile saline at body temperature were instilled up to a maximum of 150 ml, and sucked back into a sterile container. The BAL fluid obtained was used for detection of causative microorganisms. Culture for *M. tuberculosis* by Lowenstein-Jensen (LJ) solid medium was done using the BAL sediment obtained after decontamination with 4% NaOH and centrifugation at 1500 g for 10 minutes. Real time PCR of BAL fluid was performed using IS6110 insertion element as a multicopy target for molecular detection of *M. tuberculosis* complex.(8) A cut-off cycle threshold (ct) value of 40 cycles was used. Detection of other organisms (e.g. bacteria, fungi) was done by staining and culture using standard procedures.

Blood samples were taken for haemoglobin (HB), total white blood cell count (WBC), HIV test and CD4 count. Anti-HIV antibodies were detected using Capillus ™ HIV-1/HIV-2 rapid test (Trinity Biotech, Bray, Ireland) and Vironostika ® HIV uniform II ag/ab microwell enzyme immunoassay (bioMerieux, Marcy 1'Etoile, France). CD4 counting was done by flow-cytometry (Becton Dickinson Facs Count machine with BD FacscountTM reagent).
A portion of the BAL fluid was used for investigation of CMV, EBV, HSV-1/2, and HHV8; DNA extraction of the BAL samples (200 µl) was performed with the MagNA Pure LC Isolation station with the Total Nucleic Acid Isolation Kit (Roche Molecular Diagnostics, Germany) according to the manufacturer's instructions. DNA was eluted in 50 µl, and 10 µl was used for PCR. CMV DNA was detected by rt-PCR using the primers and probes previously described by Kalpoe and

collaborators,(9) on a LightCycler platform (Roche Molecular Diagnostics, Germany).

EBV and HSV-1/2 DNA were detected by rt-PCR using respectively the LightCycler EBV quantification kit and LightCycler HSV 1/2 detection kit as described by the manufacturer (Roche Molecular Diagnostics, Germany). LightCycler PCRs were set up in a final volume of 20 µl with the Fast start DNA Master Hybridization Probes Kit (Roche Molecular Diagnostics, Germany). After one step at 95°C for 10 min the samples were cycled 45 times (denaturation at 95°C for 5 s, annealing at 55°C for 10 s, and extension at 72°C for 10 s all steps with a ramp rate of 20°C/s). Fluorescence curves were analyzed with the LightCycler software, version 3.5. Semi quantitative analysis was given for EBV and CMV only, since there is no standard line available for HSV. HHV-8 DNA was detected by conventional PCR as described by Chang and collaborators.(10)

Data on the outcome of the patients were collected during the period of hospitalization and for outpatients during 4 weeks of follow up. All patients were given appropriate treatment based on the diagnosis reached by the attending team. All laboratory samples for the study were obtained prior to the start of anti-TB treatment. The study in no way interfered with the management of patients. Results obtained from this study during the course of patient's management were used in the management as needed. This study was part of the Chest Infection Study, which was conducted at the KCMC hospital. Ethical clearance was obtained from KCMC and from the Tanzanian national body governing research (NIMR). Informed written consent was obtained from every patient before any procedure was performed.

Data analysis

Using SPSS version 13 statistical package, normally distributed values were presented as mean with standard deviation (+/-SD). In other cases data were expressed as median with inter-quartile range (IQR). The McNemar's exact test and Wilcoxon Rank-sum test for related samples were used to test differences in the matched case-control data in case of dichotomous and continuous variables, respectively. For variables that were not used in the matching procedures, associations were tested for statistical significance using the Chi-square for dichotomous variables and Spearman's correlation coefficient for continuous variables, respectively. A p-value equal or less than 0.05 was regarded as statistically significant.

RESULTS

C*haracteristics of the study population*

Fifty patients were enrolled in the study; forty-three (86%) in-patients and 7 (14%) out-patients. For in-patients the median duration of hospitalization was 10 days (IQR 7-14 days). The mean age for the study population was 38 (+/- 8) years. Females comprised 54% of the study population. Seventeen (34%) of the 50 patients had fever, 29 (58%) had dyspnoea and mean oxygen saturation was 91% (+/- 9). As shown in **Table 1,** these characteristics, as well as CD4 T cell count, HB and WBC count, were similar in the two groups of patients (i.e. TB and the non-TB). Forty-seven patients (93%) had used various types of antibiotics, but not antimycobacterial agents before admission to this hospital. In the non-TB group, causative organisms were identified in 14 (56%) of the 25 patients: in 9, bacteria were cultured (2 *S. pneumoniae*, 1 *S. pyogenes*, 2 *Staph. aureus*, 2 *Ps. aeruginosa* and 2 coliform bacteria), in 2 *Pneumocystis jeroveci* and in 3

patients fungi were identified (1 *Cryptococcus neoformans*, 2 *Aspergillus fumigatus*), while in the remaining 11 patients (44%) no causative organism was identified.

Herpes viruses identified

In 41 (82%) of 50 patients, one or more human herpes virus DNA were identified in the BAL fluid, 21 in the TB group and 20 in the non-TB group (McNemar test, p = 1.0). EBV was identified in 39 (78%) patients, CMV in 12 (24%), HHV8 in 7 (14%), HSV-1 in 7 (14%), and HSV-2 in 1 (2%). Nineteen (46.3%) of the 41 patients had more than one type of HHV in the BAL fluid. Of these 19 patients, 8 were in the TB and 11 in non-TB group (McNemar test, p = 0.55). Sixteen (84.2%) of the 19 patients with multiple HHV were female (χ^2 = 4.45, p = 0.035).

EBV had the highest prevalence in both groups: 20 (80%) of 25 patients with TB and 19 (76%) of 25 patients with non-TB chest problems had positive PCR in BAL for EBV (McNemar test, p = 1.0**)**. CMV was identified in 3 (12%) of the TB patients and in 9 (36%) of the patients with non-TB infections (McNemar test, p = 0.11). HHV8 DNA was detected in 2 (8%) patients with TB and 5 (20%) patients with non-TB infections (McNemar test, p = 0.45), HSV-1 in 4 (16%) patients with TB and 3 (12%) patients with non-TB infections (McNemar test, p = 1.0), and HSV-2 was seen in one non-TB patient (**Figure 1**).

Viral load estimation for EBV and CMV

Median EBV load in the TB group was 60 copies/ml (IQR 40 – 500) and not different from that for the non-TB group, 80 copies/ml (IQR 10 – 500) (Wilcoxon Rank-sum, p=0.66). The median viral load for CMV in the TB group was 5000

copies/ml (IQR 100 – 10000) and that for non-TB group was 1000 copies/ml (IQR 300 – 7500) (Wilcoxon Rank-sum, p=0.86).

Correlation of herpes viruses identified with laboratory and clinical parameters

Ten (52.6%) of the 19 patients with multiple HHV had low WBC i.e. less than 3.5*109/l, while in the patients with single type of HHV only 3 (13.6%) of 22 patients had low WBC (χ^2 = 4.3, p = 0.017).

Mean oxygen saturation of the 12 patients with CMV was 90% (+/- 8%). Nine patients (75%) had dyspnoea and 7 (58.3%) had fever. Seven patients (58.3%) had both fever and dyspnoea.

In the non-TB group, eight of the nine (88.9%) with CMV DNA in the BAL fluid did not grow any other organism in culture of the BAL fluid.

Of the 7 cases with HSV-1 DNA, 6 had CD4 T cell count of less than 50 cells/ml, while that of the remaining case was 82 cell/ml. HSV – 1 was statistically significantly associated with CD4 T cell count less than 50 cells/ml, (χ^2 = 4.15, p = 0.042). Three of the 6 patients with CD4 T cell count less than 50/ml, had CD4 T cell less than 5 cells/ml. The patient with HSV-2 had CD4 T cell count of 36 cells/ml. No association was found between CD4 T cell count and presence of other HHV, whether single or multiple HHV in the same patient.

Seven patients had HHV8 in the BAL fluid and 8 patients had clinically detectable Kaposi's sarcoma (KS) on routine clinical examination, but only two patients had both HHV8 in the BAL fluid and clinically detected KS lesions; one KS lesion was seen on the soft palate and the other in the trachea. There was no statistically significant association between presence of KS lesions and HHV8 in BAL fluid.

Clinical outcome in relation to presence of herpes viruses

Ten out of the 50 patients (20%) died during the one-month follow-up period. Of these, 8 (32%) were TB cases and 2 (8%) did not have TB (McNemar test, p = 0.11). Of the 8 who died in the TB group, 4 were AFB smear positive and started on anti-TB treatment immediately, 2 were put on empirical anti-TB treatment on fourth and tenth day after admission, respectively. The other two were put on empirical common antibiotic treatment by the attending team pending culture results. Seven of the 8 patients who died in the TB group had at least one type of HHV DNA in the BAL fluid and the other one did not have HHV. Of the two who died in the non-TB group, one had and the other did not have HHV DNA in BAL fluid.

The presence of viral infections was not associated with fatal outcome (χ^2 = 0.34, p = 0.85) and CD4 T cell count at enrolment was not a predictor of fatal outcome in this selcted group of patients (Wilcoxon rank-sum, p=0.42).

DISCUSSION

In this study we found a very high prevalence of HHV in BAL fluid of HIV-infected Tanzanian patients presenting with pulmonary infections. The prevalence was almost two-fold higher than in a similar study conducted in Danish HIV-infected patients,(6) although the two studies were similar in terms of sample size of the study, age and CD4 T cell count of the patients. In the study conducted in Denmark, the overall prevalence of HHV in HIV-1- infected patients presenting with pneumonia was 44%.(6) In our study, the rate of multiple types of HHV of 46.3 % in one patient was also very high compared to the Danish study.(6) This showed a distinct difference in HHV burden between these two HIV study populations from two different socio-economical and environmental settings,

despite similar levels of HIV-immunosuppression. It has been speculated that recurrent malarial or other parasitic infections, may contribute to the high prevalence of HHV-DNA in respiratory samples in Africa.(11) Frequent parasitic infections are very common in sub-Saharan Africa, including in our study area. Malaria and other parasites affect the immune system and may impair HHV-specific T cell immunity and, thus, influence HHV- DNA levels.(12)

In our study the prevalence of HHV DNA in BAL fluid was equally high in both patients with TB and those with non-TB chest problems. Active *M. tuberculosis* infection in HIV-1 infection enhances replication and increases HIV-1 viral load,(13) resulting into fast progression of HIV-1 infection. We did not find significantly higher viral loads for EBV and CMV in TB patients compared with non-TB patients. This indicated that presence of HHV in BAL fluid was not influenced by *M. tuberculosis* infection and vise versa.

Of the four human herpesviruses, EBV was identified in 39 (78%) of 50 patients. This high prevalence was much higher than what was found in Denmark (5.5%).(6) A high prevalence of EBV infection in a developing country compared to a developed country was also observed in a study by Grando LJ and colleagues,(14) who identified oral EBV in 56% of Brazilian children and 27% of American children. Relatively few studies are available concerning EBV and pulmonary disease in AIDS,(6) however one could speculate that high frequency of EBV in HIV-infected patients observed in our study will increase the risk of developing EBV-related pathologies such as Kaposi's sarcoma,(15)

lymphoproliferative disorders, lymphoma, (5) and interstitial lung disease with fibrosis,(16) and worsen the long term outcome of these patients.

The clinical importance of pulmonary CMV in HIV infected patients is controversial. CMV is rarely a primary cause of pneumonia in these patients.(17) The diagnosis of CMV pneumonitis according to Tamm and colleagues is based on symptoms of fever, dyspnoea, hypoxia, and diffuse infiltrates on chest radiograph in combination with microscopic detection of CMV in the BAL fluid.(18) According to the Canadian Society of Transplantation, in transplant patients, the presence of features of pulmonary disease and evidence of CMV DNA in BAL fluid in the absence of other documented cause is defined as probable CMV pneumonia.(19) In our study all patients had signs and symptoms suggestive of respiratory infection and in most patients with CMV DNA in BAL no other organisms were found. CMV viral load however, was not high and the presence of CMV as infection or disease did not have an independent influence on the one-month outcome of the patients even without specific anti-CMV treatment. However the detection of CMV in BAL even without evidence of a disease in this study is indicative of high risk of later development of CMV disease, (20) or other complications associated with CMV e.g. other opportunistic infections, (7) and therefore long-term follow-up is needed for such patients.

Of 7 patients with HHV8 in BAL fluid, only 2 had clinically visible Kaposi sarcoma (KS) lesions on routine clinical examination. Detection of HHV8 in BAL is associated with pulmonary KS,(21) however its association with skin KS is unreliable.(22) In our patients KS lesions in the lungs may have been missed by

bronchoscopy. This is well possible in case of minor lesions located distally in the endobronchial tree or in the lung parenchyma. Lung parenchyma is the primary site for pulmonary KS,(21) although it may occur in the visceral pleura, mediastinal lymph nodes and tracheobronchial tree.(23) In our patients, presence of HHV8 in BAL fluid without clinical evidence of lung KS may indicate subclinical KS or high risk for later development of KS.(24;25) Pulmonary KS, unlike skin KS, contributes decisively to morbidity and mortality, (24) and this remains so to-date in our set up due to lack of specific treatment for KS. These patients with HHV8 in BAL fluid also have a higher risk for later development of other conditions related to HHV8 such as primary effusion lymphoma (PEL),(26) and Multicentric Castlemans' disease (MCD).(27) KS, PEL and MCD have been found to be associated with HIV infection.(5)

Herpes simplex pneumonia is extremely rare even in AIDS patients. Afessa found only one case in 1,225 consecutive hospital admissions. (28) In our study the HSV prevalence of 14% was very high compared to other studies (29;30), although we do realize that presence of HSV DNA does not necessarily mean HSV pneumonitis. Usually herpes pneumonia is due to HSV-1.(29) Studies have shown that critically ill patients with HSV-1 in BAL fluid have 40% chance of dying,(31) mainly because of severe underlying disease and co-morbidity, which may predispose to endogenous reactivation of HSV-1. For this reason, isolation of HSV-1 from lung secretions was thought to be a marker of severe illness,(31) and poor prognosis. In our study however, despite of high prevalence and association with low CD4 T cell count (which is a marker of advanced HIV/AIDS), HSV-1 was not an independent determinant of fatal outcome in one-month follow-up.

Pneumonia due to HSV-2 is even rarer.(32) A report has shown that HSV-2 pneumonia can lead to complications like pulmonary hypertension and heart failure, conditions which resolve with anti-herpetic drugs.(29) In our study the patient who had HSV-2 infection improved without specific antiviral treatment

Finally, it is difficult to rule out completely that the presence of HHV-DNA in BAL fluid was caused by contamination from the upper respiratory tract. High levels of EBV-DNA have been reported in Uganda indicating that the risk of contamination is realistic.(11) In case of contamination, however, we would have expected to detect relatively much more frequent HSV-1 DNA in the lavage fluid since shedding of this virus, even in non-immunocompromised subjects, is very common.(33)

Conclusion

In this study we found a very high prevalence of human herpes viruses in the BAL fluid of symptomatic and severely immunosuppressed HIV-infected Tanzanian patients. The prevalence of any HHV was equally high in TB and non-TB patients. Thus, we were unable to show in this relatively small study that *M. tuberculosis* enhanced reactivation and replication of HHV and we also were not able establish any influence of HHV infection to TB. In both the TB and the non-TB group, the prevalence of EBV was the highest of all HHV. Mortality in TB patients at one-month follow up was very high, however presence of HHV in BAL fluid was not an independent predictor of one-month mortality. A larger study, also using immuno-histochemical staining for HHV of BAL material is warranted to elucidate the clinical significance of HHV in HIV infected patients.

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Table 1. Characteristics of the two groups of HIV-infected patients.

Parameter	TB	Non-TB
Number of patients, N	25	25
Hospital stay in days, median (IQR)	10 (7-15)	9 (7-14)
Age, mean (SD)	38 (8)	37 (8)
M : F ratio	1 : 1.1	1 : 1.3
Oxygen saturation in %, mean (SD)	94 (6)	89 (10)
Dyspnoea, n (%)	13 (52)	16 (64)
Fever, n (%)	8 (32)	9 (36)
CD4 T cell count/mm^3, median (IQR)	47 (30 – 81)	50 (17 – 97)
HB g/l, median (IQR)	82 (65.5 – 96.0)	89(67.5 – 111.0)
WBC *10 ^{9}/l, median (IQR)	5.4 (3.1 – 9.3)	4.9 (3.3 – 6.6)

IQR: interquartile range; HB: haemoglobin; WBC: white blood cell count

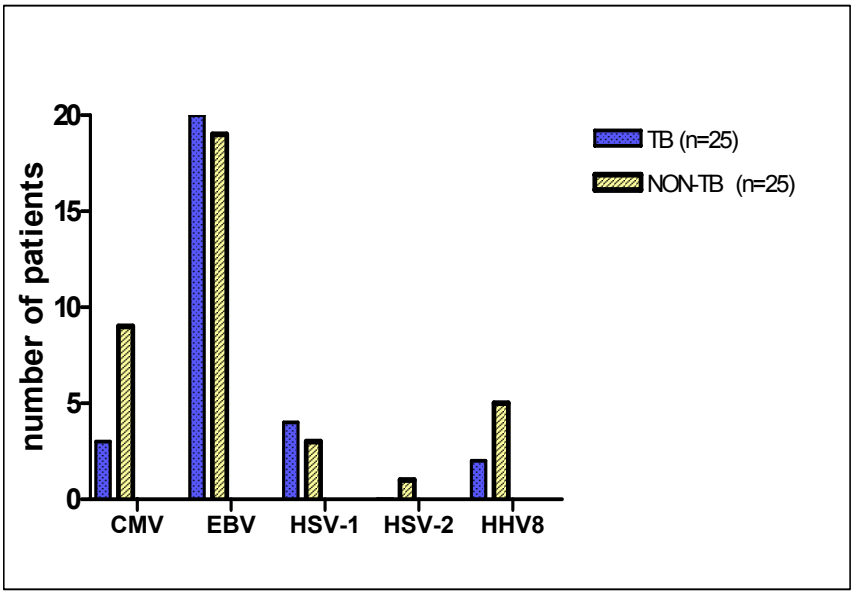

Figure 1. Number of patients with herpes viruses in BAL fluid in 25 HIV infected patients with TB and 25 HIV infected patients with non-TB pulmonary infections

Chapter 5

Laboratory diagnosis of pulmonary tuberculosis in TB and HIV endemic settings and the contribution of real time PCR for *M. tuberculosis* in bronchoalveolar lavage fluid

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ABSTRACT

Background

Tuberculosis (TB) in Africa is increasing due to the HIV epidemic and in HIV/AIDS patients it presents atypically. Pulmonary tuberculosis (PTB) in Africa is mainly diagnosed clinically, by chest radiograph or by sputum smear for acid fast bacilli (AFB).

Methods

We evaluated in 120 HIV infected patients with chest infection the diagnostic accuracy of AFB smear of sputum and bronchoalveolar lavage fluid (BAL), sputum *M. tuberculosis* (MTB) culture, real-time PCR and MycoDot® serological test, using MTB culture of BAL as gold standard. We correlated PCR cycle threshold values (C_T) to the culture results. Retrospectively, we evaluated the development of active TB in patients with positive PCR but negative culture.

Results

Culture of BAL fluid identified 28 patients with PTB. Fifty-six patients could not produce adequate sputum. Sputum AFB smear and the serological test had sensitivities of 66.7% and 0%, respectively. PCR with C_T 40 was positive in 73 patients, 27 of which were also TB culture positive (96.4% sensitivity and 52.3% specificity of PCR). PCR with C_T 32 had sensitivity of 85.7% and specificity of 90.9% to diagnose PTB in BAL. No patients with positive PCR but negative culture developed active TB during 18 months follow-up.

Conclusion

In these HIV infected patients, AFB smear and serology had very low sensitivities. PCR of BAL with C_T value 32 had improved specificity to diagnose active PTB. A prospective follow up study is warranted in TB/HIV endemic settings, applying real time PCR to both sputum and BAL.

INTRODUCTION

About one-third of the world population is infected with *Mycobacterium tuberculosis* (MTB). By 2000 there were 8.3 million new cases and up to 2.2 million deaths per year due to tuberculosis (TB) (1). Human immunodeficiency virus (HIV) infection is a potent risk factor for TB. HIV increases the risk of reactivating latent MTB (2), of rapid TB progression after infection or reinfection with MTB (3) and of raised transmission rate in the community (4). The TB burden in countries with a generalized HIV epidemic has therefore increased rapidly over the past decade, especially in the severely affected countries of eastern and southern Africa (5). In these countries up to 31% of new TB cases are attributed to HIV infection (1). In Tanzania, new TB cases increased five fold from 11,753 in 1983 to 61,603 in 2001 largely due to HIV/AIDS (6). TB has therefore become the most common disease associated with AIDS and the leading cause of morbidity and mortality (7;8). Diagnosis of pulmonary tuberculosis (PTB) in these countries depends largely on the clinical picture, radiographic findings and sputum Acid Fast Bacilli (AFB) smears (9). Largely this is due to the lack of other diagnostic facilities.

Unlike the straightforward diagnosis and typical presentation of PTB in HIV-1 seronegative individuals (10;11), the diagnosis of PTB in HIV/AIDS is more difficult. PTB in HIV/AIDS frequently has atypical clinical and radiographic presentation (11-14). Also, different causes of pulmonary infection in HIV/AIDS are often indistinguishable on the basis of clinical and radiological features (15). This increases the chances of inaccurate clinical diagnosis. Indeed, it has been found that more than 60% of AIDS patients on empirical therapy will need a change in their antibiotic treatment when the aetiology of the chest infection is eventually established (16). Therefore, for appropriate and effective treatment

an aetiological diagnosis is necessary, especially in PTB because of the high mortality associated with PTB in HIV/AIDS.

The aim of the study in this TB and HIV endemic setting was to assess the performance of different diagnostic laboratory methods: AFB smear, culture, real time PCR (PCR) using sputum and/or bronchoalveolar lavage fluid (BAL) and the MycoDot® serological test in TB/HIV co-infected patients. All patients had symptoms and signs of chest infection. Other aims were, to determine the duration of BAL culture growth, to determine the proportion of smear-negative tuberculosis, to correlate the bacillar load to duration of culture growth, to correlate the BAL TB results with cycle threshold (C_T) value in positive PCR cases, and to determine the long-term clinical significance of positive PCR when culture was negative for TB. To our knowledge this is the first study using real time PCR for TB in Sub-Saharan Africa.

MATERIALS AND METHODS

At Kilimanjaro Christian Medical Centre (KCMC) in Northern Tanzania, we enrolled 120 HIV infected patients of 18 years and above, who presented with features of chest infection: cough (dry or productive), fever, chest pain, or chest radiographic abnormalities. These patients were referred for bronchoscopy by the attending physician as part of patient's management. More than four-fifths of these patients had previous empirical antibiotic treatment for suspected pneumonia before bronchoscopy. Pregnant women and patients with oxygen saturation less than 90% under 6 l/min of oxygen were excluded from the study.

Two sputum samples (morning-spot) were collected prior to bronchoscopy. Peripheral blood samples for haemoglobin (HB), total and differential white blood cell count (WBC), HIV test, CD4 T cell count and MycoDot® serological test were taken. Using a flexible fibreoptic bronchoscope (Olympus p45 Tokyo, Japan) bronchoscopy was performed by standard procedure. Briefly, the bronchoscope was wedged into one of the heavily involved segmental bronchi as seen on the chest radiograph. Alternatively, in the case of diffuse lung involvement, the bronchoscope was wedged in one of the segmental bronchi of the middle lobe. Then aliquots of 50 ml or less of sterile saline at body temperature, up to a maximum of 150 ml in total, were instilled and sucked back into a sterile container. A portion of the BAL fluid was used for the diagnostic tests.

Sputum and BAL samples were pretreated by decontamination with 4% (w/v) NaOH and centrifugation at 1500 g for 10 minutes. The sediment was used for the different diagnostic tests. Direct smears were examined for AFB after Ziehl Neelsen (ZN) staining. MTB culture was performed using in-house made Lowenstein-Jensen (LJ) solid medium, with a maximum incubation period of 8 weeks. Reading and reporting of the results were done weekly. When colonies were seen in the LJ medium, ZN staining was done to confirm the presence of mycobacteria. For sputum smear to be positive at least one of the two samples had to be positive for AFB.

Aliquots of BAL fluid were kept at –20 ^{0}C until PCR for MTB and spoligotyping were done. DNA extraction of the BAL samples (200 µl) was performed with the MagNA Pure LC Isolation station with the Total Nucleic Acid Isolation Kit (Roche

Molecular Diagnostics, Germany) according to the manufacturer's instructions. DNA was eluted in 50 µl, and 10 µl was used for PCR.

MTB DNA amplification was performed by PCR of BAL fluid using insertion element IS6110 as a multicopy target for molecular detection of MTB complex (17). Briefly, a set of *Mycobacterium tuberculosis*-specific primers, F (GGG TAG CAG ACC TCA CCT ATG TG) and R (CCT TTG TCA CCG ACG CCT A), was used to amplify 88 bp of the IS6110-gene. Detection took place with a *Mycobacterium tuberculosis*-specific MGB-probe, FAM-labelled (TCG CCT ACG TGG CCT TT). DNA amplification/detection was done in 30 µl reaction volume with the ABI PRISM 7000. Template DNA, 333 nM of each primer and 150 nM probe. The PCR-program consisted of 2 min 50 °C, 10 min 95 °C and 45 times 15 sec at 95 °C and 1 min at 60 °C. The same primers amplify the IS6110 gene of the internal control, MSM1008. This DNA is added to see if samples contain inhibitors, which affect the efficiency of the PCR. Detection of the amplified internal control took place by a VIC-labelled TAMRA probe (CAG CTG CTA CAT GCC GGC GC). A cut-off cycle-threshold (C_T) value of 40 cycles was used.

For those who were MTB positive by BAL culture, spoligotyping was performed as described (18). Amplification of the spacers was performed using DNA extract and primers (DRa and DRb) corresponding to the direct repeat (DR) region of the genome of *M. tuberculosis.* Of the DNA extract 10 µl were mixed with 40 µl PCR mix containing 5 µl 10x PCR buffer, 3 µl magnesium chloride (25 mM), 4 µl dNTP mix (2.5 mM dNTP each), 50 pmol DRa primer, 50 pmol DRb primer, 0.2 µl Taq polymerase and 0.25 µl Tris 1 M (pH 9.0), 0.5 µl UDG (1 U/µl). Amplification and hybridization were performed according to the manufacturer's manual

(Isogen Bioscience BV, Maarssen, The Netherlands). The hybridized membrane was exposed to X-ray film for detection of the hybridization signal. The X-ray film (Hyperfilm™ ECL, Amersham Bioscience UK Ltd.) was read manually to obtain a complete pattern of the spacers between the DR regions harboured by a particular strain. Preventive measures were taken against contamination with either previously amplified DNA or amplicons from previous reactions; the procedures were described by Kwok *et al* and Longo *et al,* respectively, (19;20). Computer-assisted analysis of spoligotyping patterns was carried out with Bionumerics 4.0 (Applied Maths, St-Martin-Latem, Belgium).

Blood was tested for anti-HIV antibodies by Capillus™ HIV-1/HIV-2 rapid test (Trinity Biotech, Bray, Ireland) and Vironostika® HIV uniform II ag/ab microwell enzyme immunoassay (bioMerieux, Marcy 1'Etoile, France). CD4 T cell counting was done by flow-cytometry technique (Becton Dickinson Facs Count machine with BD Facscount™ reagent). Serum was examined using IgG-based MycoDot® assay to detect antimycobacterial antibodies against Lipoarabinomannan (LAM) antigen (MycoDot ® Mossman associates, USA) using the manufacturer's instruction manual.

We evaluated 18 months after completion of patients' enrolment the development of active TB in the subjects who were positive for MTB by PCR but negative by culture. This retrospective evaluation excluded the subjects who were given empirical TB treatment. For the remainder we looked for microbiological diagnosis of TB during this period. The case records were obtained from the Infectious Disease Clinic (IDC) of our institution where these HIV-infected patients attended. The IDC is the referral HIV centre for the

northern zone of Tanzania, where HIV infected patients attended regularly at an interval of one to three months and whenever necessary. They are monitored for HIV progression. They are examined, investigated and treated for co-infections, and started antiretroviral treatment (ARV) when indicated. The clinic is run by medical specialists and trained staff. It is supported by a range of departments including microbiology, pathology and radiology. Every visit is documented and patients' records are kept in the department of medical records.

Data analysis was done by SPSS version 10-software for Windows. Normally distributed values were presented as mean with standard deviation (SD). In other cases data were expressed as median with interquartile range (IQR). Chi-square tests were used to quantify correlation between dichotomous variables and Spearman's correlation coefficient was used to quantify correlations between continuous variables. P-value equal or less than 0.05 was regarded as statistically significant.

Clearance for the study was given by the Institutional (KCMC hospital) and National Ethical Review Boards and informed written consent obtained from each patient or a close relative.

RESULTS

General characteristics of the study population.

We enrolled 120 patients; with a mean age of 39 years (SD +/-9). The male to female ratio was 1: 1.2 and median CD4 count 65 (IQR = 20 – 147) cells/ml.

Laboratory methods for PTB diagnosis, and their sensitivity and specificity

Table 1 shows the results of different diagnostic tests. Sensitivity and specificity were calculated using BAL MTB culture as the gold standard. Twenty-eight patients (23.3%) were MTB culture positive and 18 cases (15%) smear positive for AFB from the BAL specimens. Four of the 120 samples (3.3%) had contamination. Of the 28 BAL culture positive samples, 16 were AFB positive and 12 AFB negative. Of the two remaining smear positive samples, one sample had contamination and the other was AFB positive but BAL culture negative. The sensitivity and specificity of BAL AFB smear were 57.1% and 98.9%, respectively. Sixty-four patients (53.3%) out of 120 produced adequate sputum. The remaining 56 (46.7%) either had a dry cough (43 patients, 35.8%) or had poor quality and/or inadequate amount of sputum (13 patients, 10.8%). Of the 64 patients with adequate sputum, 12 were TB culture positive (18.8%), 8 smear positive for AFB (12.5%) and 48 were TB culture negative in both sputum and BAL fluid. Two sputum samples were contaminated. The sensitivity and specificity of the 64 patients who could produce sputum were 100% and 100%, respectively for culture, and 66.7% and 100%, respectively for AFB smear.

In BAL fluid, using 40 as the C_T cut-off value of PCR, 73 samples were found to be positive for MTB; 27 of them were also BAL culture positive, 42 were culture negative and 4 samples were contaminated in culture. One culture positive sample was PCR negative for MTB and 46 samples were negative by both culture

and PCR. The sensitivity and specificity of PCR were 96.4% and 52.3%, respectively.

By using 32 as the C_T cut-off value of PCR, 34 samples were found to be positive for MTB; 24 of them were also culture positive, eight were culture negative and 2 PCR positive were contaminated (the other 2 contaminated samples were PCR negative). One culture positive sample was PCR negative for MTB and 80 samples were negative by both culture and PCR. The sensitivity and specificity of PCR (C_T value = 32) were 85.7% and 90.9%, respectively (**Table 1)**.

Two patients (1.7%) were TB positive as determined by the MycoDot® test, another 4 patients had dubious results. None of the BAL culture positive patients was positive by the serological test. The sensitivity and specificity of the serological test were 0% and 97.6%, respectively.

Correlation of BAL TB culture and AFB smear results with PCR cycle threshold (C_T) value (n = 73 positive PCR)

As seen in **Figure 1**, the median C_T value in PCR for the 27 BAL TB culture positive cases was 24 (IQR 22 - 32) and that of the 42 BAL TB culture negative cases was 35 (IQR 34 – 37). The difference was statistically highly significant (Spearman's correlation coefficient**,** $p < 0.0001$). The median C_T value for the 17 AFB smear positive cases was 24 (IQR 22 – 27) and that for the 56 negative cases 35 (IQR 32 - 37), Spearman's correlation coefficient**,** $p < 0.0001$. Both culture and smear positive results were statistically significantly associated with high MTB DNA load and negative culture results associated with low MTB DNA load as determined by C_T values in PCR.

Spoligotypes of cultured MTB strains

In 25 of the 28 BAL samples with positive MTB culture, we were able to perform spoligotyping of the strains. In all 25 the spoligotypes indicated that the isolates belong to *M. tuberculosis* complex. Sixteen of the 25 showed different patterns. Two patterns were found in 2 strains, one pattern in 3 strains and one in 5 strains.

Duration of culture growth and correlation with number of colonies in BAL culture positive samples.

Of the 28 BAL samples with positive TB culture, 6 samples (21.4%) allowed MTB to be cultured from them within four weeks, the rest 22 (78.6%) required a month or more for growth. Seventeen (60.7%) of the 28 samples grew only few colonies (i.e. a maximum of TB +1) and 11 (39.3%) samples grew more colonies (i.e. +2 and +3).

Correlation between MTB PCR / culture results and level of immunity

The median CD4 T cell count of the 27 patients positive for MTB by both PCR and culture was 47 (IQR 29 – 80) cells/µl, the median CD4 T cell count for the 42 patients with positive PCR but negative MTB culture results was 76 (IQR 18 – 186) cells/µl and the median CD4 T cell count for 46 patients with negative results by both PCR and culture was 72 (IQR 15 – 124) cells/µl. There were no statistically significant differences among these CD4 levels.

Causative agents identified and retrospective review of outcome after18 months in patients with positive TB PCR and negative BAL TB culture.

Of the 46 patients who were PCR positive but culture negative, 14 patients (30%) were put on empirical anti-TB treatment (before PCR results were known) of which three died. Clinical follow-up data were retrieved of the 32 patients who did not receive empirical TB treatment: 4 of these patients died later on subsequent admissions while the causes of death were not established. The case records of the remaining 28 patients were retrieved from the Infectious Disease Clinic where these patients were still attending during a period of 18 months or more after the PCR for tuberculosis was found to be positive. In 14 of them the following causative organisms were found either in BAL or in sputum: common bacteria in 10 (six *Streptococcus pneumoniae*, two *Staphylococcus aureus* and two coliform bacteria); *Pneumocystis jiroveci* in two; cryptococcus in one and *Human Herpes Virus 8* in one. In the remaining 14 patients no causative organisms were identified. Fifteen of the 28 patients (53.6%) were on highly active antiretroviral therapy (HAART) at that time. Despite not receiving anti-TB treatment, none of the 28 patients was diagnosed to have active tuberculosis during this period.

DISCUSSION

We found that the microbiological diagnosis of PTB in HIV infected patients by the existing traditional methods was inefficient. Sputum AFB smear had limited ability to diagnose PTB. This is a serious problem given the resource limitation, lack of other diagnostic options and the endemic nature of both TB and HIV in Tanzania. The low sensitivity of sputum AFB smear for PTB we observed is similar to other studies (21-23). In our study however, the low sensitivity of AFB

smear for PTB was coupled with the inability to produce adequate sputum in nearly 50% of patients. Sputum-scarcity in HIV infected individuals (especially with low CD4 counts) hampers the diagnosis of PTB by conventional AFB smear (24). For AFB smear to become positive it requires 5000 – 10000 AFB per ml (25). Therefore sputum-scarcity and the paucibacillary nature of TB in HIV infection (26), can result into misdiagnosis or into classifying patients as smear-negative PTB. In this study nearly three-quarter of the BAL culture confirmed TB were either sputum smear negative or did not produce sputum. Smear negative PTB in HIV/AIDS patients more often than smear positive presents with atypical picture; both clinically and radiographically (from normal chest radiography to slight or atypical changes) (27;28), which makes the diagnosis of PTB in this group even more difficult. Apart from their role in the transmission of MTB (29), in smear-negative patients the diagnosis of PTB is delayed, which resulted in increase in mortality (30-32).

Although culture was more sensitive than AFB smear, culture growth was slow; most samples did not show visible colonies of MTB before one month, thus delaying the diagnosis.

The MycoDot® serological TB test which is cheap, rapid and technically not very demanding was extremely insensitive in this study of HIV/AIDS patients, while it has been shown to be fairly sensitive and highly specific in HIV seronegative patients (33). In our study the specificity of MycoDot was also very high. Unlike the sensitivity, the specificity of MycoDot has been shown not to be influenced by HIV infection (33;34). Sensitivities as low as 3% has been reported previously (34). The extremely low sensitivity noted in this study may be attributed to the low median CD4 T cell count, since the sensitivity of MycoDot test is directly proportional to CD4 T cell profile (33).

Real time PCR which is faster, but technically more demanding and expensive was found to be highly sensitive in this study. High sensitivity of PCR has also been reported by a study using conventional PCR on sputum samples from Kenya, another TB and HIV endemic area (35). The higher sensitivity demonstrated in our study than that found in Kenya may also be attributed to the use of BAL fluid instead of sputum. It has been found that the use of BAL improves sensitivity compared to use of sputum (36). DNA contamination is a possible cause of false positive results in PCR methods. However compared to conventional PCR, real time PCR lowers the chance of contamination during sample processing substantially and reduces both false positive and false negative results. Also, we performed all molecular tests in three separate rooms: a clean room for preparing mixtures, one room for DNA isolation and one room for DNA amplification, thus reducing the chance for DNA contamination considerably. Also the use of uracil as a mix component is a substantial improvement in the prevention of DNA contamination. Adding uracil to the mixture will break down all DNA strands containing UTP (produced by amplification in the laboratory), before starting the PCR program.

Low specificity due to the decontamination technique used for culturing is also unlikely, since the contamination rate was within normal ranges. The statistically significant correlation we observed (Figure 1) between MTB culture positive results with low C_T values (i.e. high DNA load) and culture negative with high C_T value in this study also argues against the influence of a harsh decontamination technique. Harsh decontamination technique more likely would have resulted in culture negative but positive PCR with low C_T value. Low specificity therefore may be partly explained by recent history of TB treatment and the exquisite sensitivity of the PCR technique (17;37).

Lowering the C_T values of PCR demonstrated a better correlation with MTB culture results. Of 28 MTB positive cultures, 24 had a C_T value equal or less than 32 while of 88 MTB negative cultures, 80 had a C_T value above 32, resulting in a sensitivity of PCR of 85.7% and specificity of 90.9% (Table 1) These findings imply that a C_T value of 32 and below was indicative of active TB and that a C_T value above 32 indicated a mere presence of TB DNA, but not active disease. Therefore PCR in BAL with 32 as a cut off C_T value may be useful as a rapid method for diagnosis of active tuberculosis.

Latent MTB infection is common in TB endemic regions where the first mycobacterial infection is acquired during childhood and is kept under control by the immune system resulting into only about 10% of them developing the disease in their lifetime (38). Even when the infection is successfully controlled by the immune system, some bacilli will remain in the tissues in a latent state (39). In a study of PCR applied to superficially normal lung tissue obtained at necropsy from patients who had died from causes other than TB in Ethiopia and Mexico, up to 38% of samples tested were positive for MTB, whereas none of the samples from Norwegian individuals were PCR positive (40). In this study, alveolar macrophages were found to be the most common cells with positive mycobacterial DNA labelling. Based on these findings and because the alveolar macrophages are easily accessible at bronchoscopy by BAL, we postulate that, in view of the high endemicity of tuberculosis in Tanzania, the high percentage of samples with positive mycobacterial DNA in the absence of active PTB, actually may represent latent TB in these patients.

In conclusion, we found that around half of HIV infected patients with symptoms and signs of PTB do not produce (adequate) sputum, which makes microbiological diagnosis difficult. But even in those who manage to produce

sputum, microbiological diagnosis of PTB using the WHO recommended method for resource-limited settings is inefficient. As a consequence many PTB cases are missed and therefore not treated appropriately. The serological test had very limited value in TB diagnosis in HIV infected patients due to very low sensitivity. Culture was limited by prolonged duration of mycobacterial growth. Real time PCR of BAL was limited by low specificity despite high sensitivity, although specificity increased to over 90% when a cut off point of 32 for the C_T value in PCR was adopted. A prospective follow up study of PCR is warranted in a TB/HIV endemic resource-poor setting, applying real time PCR to both sputum and BAL.

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Table 1. Comparison of different *Mycobacterium tuberculosis* (MTB) diagnostic tests, and sensitivity and specificity of each, using BAL MTB culture as the gold standard

		BAL MTB CULTURE RESULTS				Sensitivity	Specificity
		Positive	Negative	Contamination	TOTAL	(%)	(%)
BAL AFB smear	Positive	16	1	1	18	57.1	98.9
	Negative	12	87	3	102		
	TOTAL	28	88	4	120		
Sputum culture	Positive	12	0	0	12	100	100
	Negative	0	48	2	50		
	Contamination	0	1	1	2		
	TOTAL	12	49	3	64		
Sputum AFB smear	Positive	8	0	0	8	66.7	100
	Negative	4	49	3	56		
	TOTAL	12	49	3	64		
BAL PCR (C_T 40)	Positive	27	42	4	73	96.4	52.3
	Negative	1	46	0	47		
	TOTAL	28	88	4	120		
BAL PCR (C_T 32)	Positive	24	8	2	34	85.7	90.9
	Negative	4	80	2	86		
	TOTAL	28	88	4	120		
Serological test	Positive	0	2	0	2	0	97.6
	Negative	27	83	4	114		
	Dubious	1	3	0	4		
	TOTAL	28	8	4	120		

Sensitivity and specificity of PCR in BAL are given for two C_T cut off values: 40 and 32. Using the latter, specificity of PCR improved significantly.
AFB: acid fast bacilli; BAL: bronchoalveolar lavage; PCR: real time PCR

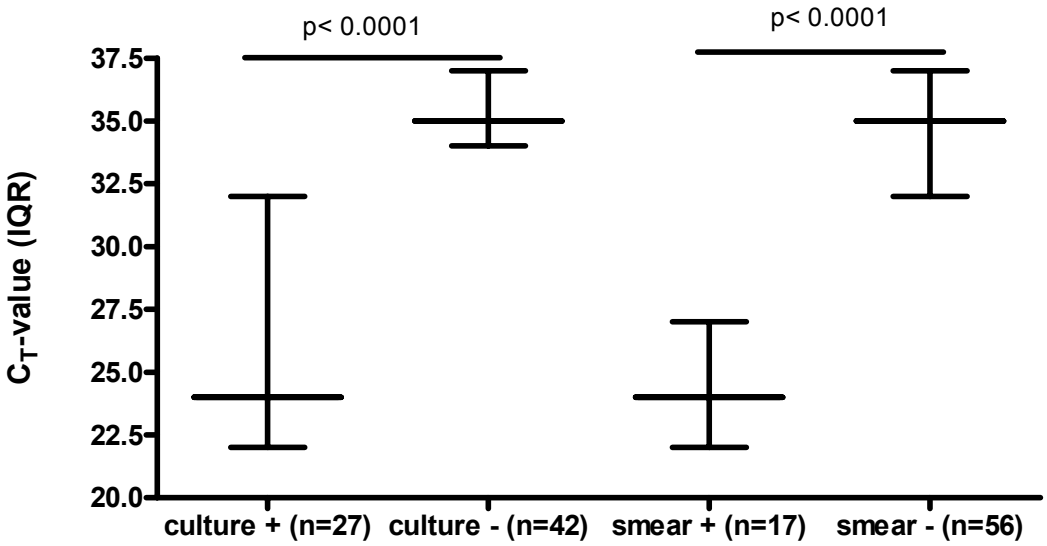

Figure 1. Differences in median C_T values (IQR) of real time PCR between positive and negative results of culture and AFB smear of BAL fluid**.**

Chapter 6

M. tuberculosis genotypic diversity and drug susceptibility pattern in HIV- infected and non-HIV-infected patients in northern Tanzania

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ABSTRACT

Background

Tuberculosis (TB) is a major health problem and its increase is largely due to HIV infection. TB and HIV are endemic in sub-Saharan Africa. Determination of the prevalent *M. tuberculosis* strains and their drug susceptibility is important for TB control.

Methods

TB positive culture respiratory samples from 130 patients were genotyped. The spoligotypes were correlated with anti-tuberculous drug susceptibility in HIV-infected and non-HIV patients from Tanzania.

Results

One-third of patients were TB/HIV co-infected. Forty-seven spoligotypes were identified. Fourteen isolates (10.8%) had new and unique spoligotypes. The major spoligotypes contained nine clusters: CAS1-Kili 30.0%, LAM11- ZWE 14.6%, ND 9.2%, EAI 6.2%, Beijing 5.4%, T-undefined 4.6%, CAS1-Delhi 3.8%, T1 3.8% and LAM9 3.8%. Twelve (10.8%) of the 111 phenotypically tested strains were resistant to anti-TB drugs. Eight (7.2%) were monoresistant strains: 7 to isoniazid (INH) and one to streptomycin. Three strains (2.7%) were MDR and one (0.9%) was resistant to INH and streptomycin. Mutation in the *kat*G gene codon 315 and the *rpo*B hotspot region showed a low and high sensitivity, respectively, as predictors of phenotypic drug resistance.

Conclusions

CAS1-Kili and LAM11-ZWE were the most common families. Strains of the Beijing family and CAS1-Kili were not or least often associated with resistance, respectively. HIV status was not associated with spoligotypes, resistance or previous TB treatment.

INTRODUCTION

Tuberculosis (TB) persists as a major cause of morbidity and mortality, affecting almost a third of the world's population(1). Inadequate detection and cure rates have been identified as reasons for a mounting global tuberculosis burden (2). Human immunodeficiency virus (HIV) is by far the major cause of the current increase in tuberculosis infection. The presence of HIV increases the risk of reactivation of a latent Mycobacterium tuberculosis (MTB) infection (3) and rapid thus progression of the infection (4); HIV also increases MTB transmission rates at the community level, therefore threatening the health and survival of HIV-seronegative individuals as well (5). Sub-Saharan Africa is endemic for both TB and HIV infection (6), and pulmonary tuberculosis (PTB) in the HIV-affected countries of eastern and southern Africa, such as Tanzania, has increased rapidly in the past decades (7).

Molecular genotyping is an important tool for the understanding of TB epidemiology. It can predict transmission rate and identify dominant strains, strains with an enhanced capacity to spread, strains associated with outbreak (8), severe disease (9) and drug resistance. Spacer oligonucleotide typing (spoligotyping) is one of the molecular genotyping techniques; it is fast, robust, reliable, easy to perform, and cost-effective (10). On the basis of the variability of the direct-repeat (DR) locus (10;11), spoligotyping can detect different M. tuberculosis strains, such as the Beijing genotype which is associated with enhanced febrile response in patients during treatment, and multiple drug resistance (MDR) (9;12;13).

For TB control, monitoring the emergence of drug resistant strains is essential. While detection of drug resistance by phenotype is hindered by the prolonged time to identify resistant strains, genotypic prediction of drug resistance is faster. Data are accumulating on the correlation between gene mutations and phenotypic resistance. Such data however are sparse, particularly from sub-Saharan Africa where the disease burden is highest. The primary mechanism for acquiring resistance in M. tuberculosis is the accumulation of point mutations in gene coding for drug targets or drug-converting enzymes (14). Mutations in the catalase peroxidase gene (katG) (15;16) and in a gene encoding the enoyl acyl carrier protein reductase (inhA) (17) have been found to account for 60 to 70% and 10 to 15% of INH-resistant MTB isolates, respectively (18). Mutations resulting in an amino acid change within the 81-bp core region of the RNA polymerase β-subunit (rpoB) gene are found in 96% of rifampicin-resistant M. tuberculosis strains (19).

The objectives of this study were to determine the genetic biodiversity of MTB by spoligotyping and to correlate spoligotypes and anti-tuberculous drug susceptibility in HIV-infected and non-HIV patients in northern Tanzania, one of the TB and HIV endemic countries of eastern Africa.

MATERIALS AND METHODS

In the Kilimanjaro region of northern Tanzania, between April and September 2005, we collected sputum or bronchoalveolar lavage (BAL) fluid by direct expectoration or bronchoscopy, respectively, from 220 patients suspected of having PTB. The patients presented with coughing, evening fevers and abnormal chest x-rays. The patients were seen at Kibong'oto National Tuberculosis

Referral Hospital (KNTH) and Kilimanjaro Christian Medical centre (KCMC), a tertiary care hospital. The specimens were obtained prior to initiation of TB treatment. For each patient, any history of TB treatment in the past was noted. Specimens were digested and decontaminated with NaLC and 2%NaOH by the standard procedure (20). Sediments were used for diagnosis of TB: direct smears for acid-fast bacilli (AFB) by the Ziehl-Neelsen (ZN) method (20) and cultures in Lowenstein-Jensen (LJ) solid medium in three slants with or without 0.75% glycerol or with 0.6% sodium pyruvate. One hundred and thirty TB-positive culture samples (confirmed by smear) were subjected to spoligotyping and genotypic testing for drug resistance to isoniazid (INH) and 22 for rifampicin; 111 samples underwent phenotypic DST because the reculture of nineteen strains was not possible. Phenotypic drug resistance testing was performed for INH, rifampicin, ethambutol, and streptomycin and genotypic testing was carried out for a mutation in the katG 315 codon to INH and for a mutation in the rpoB hotspot region to rifampicin. Genotypic resistance to rifampicin was tested with samples which were INH-resistant, contained phenotypic rifampicin-resistant strains, or had selected spoligotypes; phenotypic rifampicin -sensitive strains were used as controls.

DNA extraction

A loopful of Mycobacterial colonies from cultures was suspended in 1 ml of physiological saline solution and heated at 80 ^{0}C for 10 minutes to kill the bacilli. The cell debris was removed by centrifugation at 13,000 x g for two minutes. The supernatant obtained was stored at -20 ^{0}C until used for DNA amplification (21).

Spoligotyping

Amplification of the spacers was performed using DNA extract and primers (DRa and DRb) corresponding to the direct repeat (DR) region of the genome of M. tuberculosis according to the procedure described by v.d. Zanden et al (22). Briefly, 10 µl of the DNA extract were mixed with 40 µl PCR mix containing 5 µl 10x PCR buffer, 3 µl magnesium chloride (25 mM), 4 µl dNTP mix (2.5 mM dNTP each), 50 pmol DRa primer, 50 pmol DRb primer, 0.2 µl Taq polymerase and 0.25 µl Tris 1 M (pH 9.0), 0.5 µl UDG (1 U/µl). Amplification and hybridization were performed according to the manufacturer's manual (Isogen Bioscience BV, Maarssen, The Netherlands). The hybridized membrane was exposed to X-ray film for detection of the hybridization signal. The X-ray film (Hyperfilm^{TM} ECL, Amersham Bioscience UK Ltd.) was read manually to obtain a complete pattern of the spacers between the DR regions harbored by a particular strain. Preventive measures were taken against contamination with either previously amplified DNA or amplicons from previous reactions; the procedure was described by Kwok et al. and Longo et al; respectively (23;24). Computer-assisted analysis of spoligotyping patterns was carried out with Bionumerics 4.0 (Applied Maths, St-Martin-Latem, Belgium).

The patterns obtained received a spoligo-international type (SIT) according to the cluster assignment after the sequences were processed with the International Spoligotype Database of the Institut Pasteur de Guadeloupe, (http://www.pasteur-guadeloupe.fr/tb/spolDB4) (25). New spoligotypes identified in the study were added to the SpolDB4.

Phenotypic drug resistance testing

The proportional method for drug susceptibility testing (DST) of M. tuberculosis, as described in the manual, was used (26); Briefly, for each drug a 1: 10 dilution of standardized inoculum was inoculated onto the drug free control and drug-containing media. The extent of growth in the absence or presence of drug was compared and expressed as a percentage. If growth at the critical concentration of a drug was >1%, the isolate was considered to be clinically resistant. 7H10 agar with 0.2 and 1 mg/l isoniazid (INH), 1 and 5 mg/l rifampicin, 5 and 10 mg/l ethambutol and 5 and 10 mg/l streptomycin were used. 7H10 agar tubes with 1 ml of culture in 9 ml sterile aquadest without antibiotics served as growth control. Reading was only performed if at least 50 CFU of M. tuberculosis growth appeared. Ten strains from the Dutch International Reference Laboratory for Mycobacteriology were used as controls: three M. tuberculosis strains sensitive to INH, rifampicin, ethambutol and streptomycin, and seven strains variably resistant to the four used first line anti-TB drugs.

Genotypic INH and rifampicin resistance testing

Amplification of katG gene containing codon 315 was performed for detection of INH resistance, using a set of primers; Foward (GGG CTT GGG CTG GAA GAG) and reverse (ACA ACA GTT TCC TCG AGA TCC TGT) (27). This resulted in a 122 bp long product. Detection of the katG-315 wildtype took place by a FAM-labeled probe (5’ CGCGATCACCAGCGGCATCG 3’) and detection of the most common katG-315 mutation from AGC to ACC by a VIC-labeled probe (5’CGC GAT CAC CAC CGG CAT CG 3’) by real time PCR. DNA amplification/detection was performed in 30 μl reaction volume with the ABI Prism® 7000 Sequence

Detection System (Applied Biosystems): 15 ul TaqMan® Universal PCR Master Mix (AppliedBiosystems) template DNA, 400 nM of each primer and 200 nM of each probe. The real time PCR-program consisted of 2 min at 50^0C, 10 min at 95 ^{0}C, 45 cycles of 15 seconds at 95 ^{0}C, 1 min at 60^0C.

For the detection of rifampicin resistance, sequencing was carried out with the 3100-Avant sequencer (Applied Biosystems). Amplification of a 437 bp fragment, containing the rpoB-hotspot region, took place with a PTC 200 thermocycler (Biozym) by forward primer rpoB-F1 (5'TGGTCCGCTTGCACGAGGGTCAGA3') and reverse primer rpoB-R1 (5' CGATCACACCGCAGACG3'), as described by v.d. Zanden et al. (19). The reaction mixtures in a final volume of 55 µl contained 10 µl Q-buffer from Qiagen, 5.5 µl Taq buffer (10 mM Tris, 1.5 mM $MgCl_2$, 50 mM KCl), 50 pmol of primers rpoB-rev1 and rpoB-for2, 0.23 mM dNTPs, 1U SuperTaq and 50 ng DNA and 0.22 µg TaqStart. The cycling parameters includes an initial denaturation at 96°C for one min followed by one min at 93°C, primer annealing at 63°C – 60°C for one min (2 cycles at each 1°C decrement) and one min for primer extension at 68°C. This was followed by 20 subsequent cycles of amplification; 93°C for one min, 60°C for one min 68°C for one min, followed by an additional cycle of 5 min at 68°C to complete the elongation of the PCR intermediate products.

PCR products were checked for integrity on ethidium bromide agarose gels and purified with the Qiaquick PCR purification kit (Qiagen GmbH, Hilden, Germany). For DNA sequencing reactions, the fluorescence-labeled dideoxynucleotide technology with the protocol of the manufacturer was used (Applied Biosystems, Foster City, USA). Unincorporated dye terminators were removed with MilliPore Multiscreen Assay System (Millipore, Molsheim, France) according to

manufacturer's protocol, the reaction products were separated and detected with an ABI Prism 3700 automatic DNA sequencer (PE biosystems). Both strands of the polymorphic region of the rpoB gene were sequenced.

The results of sequencing of test strains were compared with the rpoB-hotspot wildtype using Bionumerics software (Applied Maths, St-Martin-Latem, Belgium) to detect any mutation.

Blood was collected from all patients for detection of anti-HIV antibodies using the Capillus ™ HIV-1/HIV-2 rapid test (Trinity Biotech, Bray, Ireland) according to the manufacturers instructions,with positive and negative controls for each test run. Samples positive in the rapid test were confirmed by ELISA using Vironostika ® HIV uniform II ag/ab microwell enzyme immunoassay (bioMerieux, Marcy 1'Etoile, France). CD4 counts were determined using flow-cytometry technique (Becton Dickinson Facs Count machine with BD Facscount™ reagent).

Data analysis was done by SPSS version 10-software for Windows. Normally distributed values were presented as mean plus standard deviation (SD). In other cases data were expressed as mean plus standard error mean (SEM). Chi-square was used to express correlations between dichotomous variables and Spearman's correlation coefficient, to quantify correlations between continuous variables. P-value equal to or less than 0.05 was regarded as statistically significant.

Ethical clearance was obtained from the Research Ethics Committee of KCMC hospital and a signed informed written consent was obtained from patients prior to enrolment of the patients in the study.

RESULTS

Characteristics of the study population

In total 130 positive-culture samples from 220 patients were included in the study. The majority of the patients in this study were from the Chagga (41.1%) and Masai tribes (11.6%). Other tribes were represented by less than 5% each. Fifty-four (41.9%) were peasants, 28 (21.7%) business people, 18 (14%) miners and 10 (7.8%) herders, the remaining had other occupations. The mean age (SEM) was 37 years (1). Eighty-three (63.8%) were male patients. Fourteen patients (10.8%) had a history of previous treatment for tuberculosis. Forty-seven (36.2%) patients were HIV seropositive. Mean CD4 cell count (SEM) for the HIV seropositive patients was 200 (35) cells/ml and for the HIV seronegative group the mean CD4 count (SEM) was 555 (38) cells/ml.

Spoligotypes

A total of 47 spoligotypes was identified among the 130 M. tuberculosis isolates. Fourteen isolates (10.8%) had new and unique spoligotypes (SIT 0) while 116 isolates (89.2%) belonged to 33 previously known spoligotypes. A total of 106 isolates (81.5%) from nine different phylogenetic clusters formed the major isolates (i.e. with five or more isolates for each spoligotype). These were CAS1-Kili with 39 (30%) isolates (strains 60-98), LAM11- ZWE 19 (14.6%) (strains 0-19), ND 12 (9.2%)(strains 119-130), EAI 8 (6.2%)(strains 47-54), Beijing 7 (5.4%)(strains 109-115) and T-undefined 6 (4.6%)(strains 41-46) as well as CAS1-Delhi (strains 99-103), T1 (strains 31-35) and LAM9 (strains 20-24) with 5 isolates (3.8%) each (Figure)**.**

Three new shared spoligotypes (a common pattern shared by two or more isolates) were established from our findings by comparing them with the

spoligotypes which were available in the SpolDB4: one EAI (SIT 2482, strain 49) with that found in Zambia, one T-undefined (SIT 2482, or SIT 2484, strain 42) with that found in the USA and one CAS1 variant – undefined (SIT 2485, strain 107) with that found in Sweden.

Drug susceptibility testing (DST)

Out of 111 TB isolates which were subjected to DST, 12 (10.8%) were resistant to at least one anti-TB drug. Eight (7.2%) of the 111 were monoresistant strains: 7 resistant to INH and one to streptomycin. Four (3.5%) strains were resistant to multiple drugs: one (0.9%) was resistant to INH and streptomycin and the other three (2.7%) strains were MDR strains: two (1.8%) strains were resistant to all four anti-TB drugs and one (0.9%) strain was resistant to INH, rifampicin and ethambutol. Resistance of the 111 isolates for each drug was as follows: INH 11 (9.9%) strains, streptomycin 4 (3.6%), rifampicin 3 (2.7%), and ethambutol 3 (2.7%).

Four of the 130 (3.1%) MTB isolates were found to have a mutation in a katG gene codon 315. Four of the 22 tested isolates were found to have a mutation in the hotspot region of the rpoB gene. Of the 11 strains phenotypically resistant to INH, four strains were also genotypically resistant to INH. The resistant strains were phenotypical retested at the Dutch Mycabacteriology Reference Laboratory showing the same results. The sensitivity of the mutant katG gene to predict phenotypical resistance to INH was 30.8% and the specificity was 100%.

All three strains phenotypically resistant to rifampicin were also genotypically resistant to rifampicin. Both sensitivity and specificity of a mutation in the rpoB gene to predict phenotypical resistance to rifampicin was 100%.

As shown in the Table, two of the 49 isolates (4.1%) belonging to four variants of the CAS1 family (CAS1-Kili, CAS2, CAS1-undefined, CAS1-Delhi) showed resistance to at least one drug and four could not be tested. Six of the 30 isolates (20%) of the LAM family (LAM3 or S, LAM9, LAM11-ZWE) showed resistance to one or more anti-TB drugs. Three of the 16 isolates (18.8%) of the T family (T3-UGA, T1, T-undefined) showed resistance. One of the 13 isolates (7.7%) of the EAI family (EAI, EAI-variant, EAI-undefined), one of the 12 isolates (8.3%) of the ND family, and one of the three isolates (33.3%) from the MANU family (MANU1, MANU2) showed resistance to at least one anti-TB drug. None of the seven M. tuberculosis Beijing isolates was resistant to anti-TB drugs. Out of the 12 resistant isolates two (14.3%) were new and unique isolates (strain 116 and 126): the three MDR isolates were CAS1- Kili (strain 89), EAI (strain 48) and LAM 11 – ZWE (strain 13) (Figure)

Previous TB treatment, HIV status and drug resistance.

Out of 14 patients who had a history of previous treatment for PTB, one (7.1%) had a MDR-MTB strain and the other 13 (92.9%) had strains sensitive to all four first line anti-TB drugs. Five of the 47 HIV-infected individuals (10.6%) showed resistance to at least one drug. Three of the 47 HIV – infected individuals (6.4%) had a history of previous treatment for TB and none of them had strain resistant to anti-TB drug.

DISCUSSION

There was a wide diversity of spoligotypes in this study group; the 130 MTB isolates produced 47 different spoligotypes. The clades observed in this study were more than three-quarters of the total of 62 clades/lineages currently

documented in the fourth international spoligotyping database, SpolDB4 (25). This diversity may be attributed to increased human movement in this area due tourism, mining, asylum seeking (refugees) and transborder/international business. Historically, many people from Europe and Asia also lived here during pre- and post-independence times. The structure of the TB population is determined by geography, demography, and human migration. Studies have shown that the host's geographical origin is predictive of the clinical M. tuberculosis isolates and there is an apparent stable association of TB bacilli populations with their human hosts in various environments (28). New types (i.e. orphan spoligotypes) accounted for one-tenth of all spoligotypes in this study. The low percentage of new types may also be a result of increased human movements. Countries with a history of isolation have been shown to have a large number of new spoligotypes (8). In this study two of the three new shared spoligotypes were formed between two geographically widely separated isolates (strain 42 and 107). This again is more likely to be due to the increased human movement than homoplasy (i.e. acquisition of two similar structures without a common ancestor). If it is assumed that tuberculosis may have affected the early hominids in East Africa, evolved clonally and then spread to the rest of the world coincidentally with human migration out of Africa (10;11;28), then it is interesting to find that almost all strains go back to the origin of *Homo sapiens sapiens*. The site of this study is within the area of Olduvai Gorge in northern Tanzania, which is regarded as the "cradle of mankind". In this context, strains no. 116-118 with MANU-derived spoligotypes are of particular interest (Figure).

Among the different phylogenetic clusters found in this study, there were only nine major clusters with five or more isolates, but they comprised 81.5% of all

isolates. This indicates the high transmission rate within the population. This high transmission rate may also be influenced by the marked prevalence of HIV infection which increases the risk of tuberculosis for all people in a community, both those with HIV infection and the HIV seronegative community members (5). Two clusters were by far the most prevalent: CAS1-Kili and LAM11-ZWE family, with a prevalence exceeding ten percent. The predominance of these two families by more than ten percent has been demonstrated by another study based on IS6110-restriction fragment length polymorphism (RFLP). The study showed that the Kilimanjaro and Meru families were the most prevalent in this area (29), the names of the families being derived from the two adjacent mountains found in this area. While the Kilimanjaro family refers to CAS1-Kili, the Meru family is identical to LAM11-ZWE (25). Another recent study, performed in Dar-es-Salaam, suggested the existence of new CAS1-Kili genotype variants (designated as CAS1-Dar) (30). However, the absence of spacer 2 and 15 in the spoligotype-signatures reported by these authors was not found, even once, in our study. A single clinical isolate, strain 104 (ST247) looks similar to the CAS-Dar variants. This may be explained either by a specific loss of spacers 2 and 15 in the CAS1-Dar variants or by false negative hybridization spots. Spacer 2 and 15 may indeed sometimes provide barely interpretable results. If confirmed, the absence of spacers 2 and 15 may provide interesting clues about the microevolution of CAS1-Kili in Tanzania.

More than 5% of cases were Beijing isolates which were the fifth most frequent spoligotype. The presence of seven Beijing isolates had already been reported in Dar-es-Salaam (30). The mean age of the patients with the Beijing family was the lowest compared to those with other isolates (Table). It has already been

reported that Beijing is associated with young age (31) which implies recent and ongoing transmission.

A striking finding was that not one single case of M. bovis or M. africanum was found in this study. This study cohort consisted mainly of peasants and herders, including the Masai people who are essentially semi-nomads or herders and their staple food includes milk and meat. In Uganda which is a neighbouring country, other genotypes of the M. tuberculosis complex, previously designated as "M. africanum 2" are the major cause of human tuberculosis (32). This finding may be a result of improved livestock and animal husbandry in Tanzania or due to anthropologically rooted differences between Uganda and Tanzania.

All resistant isolates except one were resistant to INH, either alone or in combination with other drugs. The level of INH resistance was high; a similar trend has been observed in many African countries; in some countries up to one-third of the isolates is resistant to INH (33-36). This rate is alarming, especially since INH is the first-line drug, which is used throughout the course of TB treatment. This also means a high probability of the development of MDR in the future, since it has been observed that MDR often develops from initial INH-monoresistant strains (35). Several reports show a low prevalence of MDR-MTB in Africa compared to Asia and Eastern Europe (37). But the trend seems to be steadily increasing since previous reports from Tanzania showed that INH monoresistance was about 5% and MDR was about 1% (38) while our study reveals a more than two-fold increase in both INH resistance and MDR. The upward trend in resistance has also been noted in neighboring countries (37;39).

Despite its high prevalence, the CAS 1 family showed the least association with anti-TB drug resistance (Table); family LAM and T were more frequently associated with anti-TB drug resistance.

Another interesting aspect was the fact that all MTB Beijing isolates in this study were susceptible to all anti-TB drugs unlike reports from other studies (35;40). A previous study in Indonesia also showed no significant association between Beijing strains and drug resistance (9).

One-third of the study population was infected by both HIV and TB; this is the common trend in sub Saharan Africa (41). The association of HIV infection, however, with previous treatment for TB was not statistically significant and that with anti-TB drug resistance was absent in this study. The smaller number of HIV- infected patients with a history of previous treatment for TB may be attributed to high mortality associated with the first MTB infection in TB/HIV co-infected patients (42;43). There was no association between HIV status and spoligo patterns except for Beijing whereby only one person was HIV seropositive.

The mean CD4 count for patients with Beijing was the lowest, although all patients except one were HIV seronegative. This low CD4 count despite being HIV seronegative may be due to the pronounced virulence of the Beijing strain. It has been demonstrated that the Beijing family is more virulent than other M. tuberculosis strains. The virulence is attributed to the ability of the strain to produce phenolic glycolipid which is able to interfere with host immunity (44)

In conclusion, in this study we found a wide diversity of spoligotypes with predominance of two genotype families. The Beijing family was also among the

prevalent phylogenetic cluster. The most prevalent cluster CAS1-Kili and the most virulent Beijing were not or barely associated with anti-TB drug resistance. A substantial proportion of resistant isolates was found, INH monoresistance being the highest and thus signaling the danger of increasing MDR in the future. MDR was higher than that described in previous reports from Tanzania.

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Table. Distribution of seven of the phylogenetic families by age, smear results, HIV status, CD4 count (n=130) and phenotypic resistance for anti-TB drugs (n = 111)

Family	Isolates N=130		Age N=130		Smear positive N=130		HIV positive N=130		CD4cell/ml N=130		Resistant N=111	
	%	n/N	mean	SEM	%	n/N	%	n/N	mean	SEM	%	n/N
CAS *	37.7	49/130	38	2	81.6	40/49	34.7	17/49	471	64	4.4	2/45
LAM **	23.2	30/130	39	3	83.3	25/30	26.7	8/30	437	55	18.5	5/27
T ***	12.3	16/130	35	2	81.3	13/16	50.0	8/16	436	92	14.3	2/14
EAI #	10.0	13/130	39	3	69.2	9/13	46.2	6/13	432	64	8.3	1/12
ND	9.2	12/130	37	4	58.3	7/12	41.7	5/12	413	99	16.7	1/6
Beijing	5.4	7/130	29	2	85.7	6/7	14.3	1/7	380	69	0.0	0/6
MANU ##	2.3	3/130	32	4	100	3/3	66.7	2/3	195	141	100	1/1

* CAS: CAS1-Kili; CAS1-Delhi; CAS1-undefined ;CAS2

** LAM : LAM11-ZWE ; LAM9 ; LAM3 or S ; LAM-undefined

*** T: T-undefined; T1; T3-UGA; T3

EAI : EAI ; EAI-variant ; EAI-undefined

MANU: MANU1; MANU2.

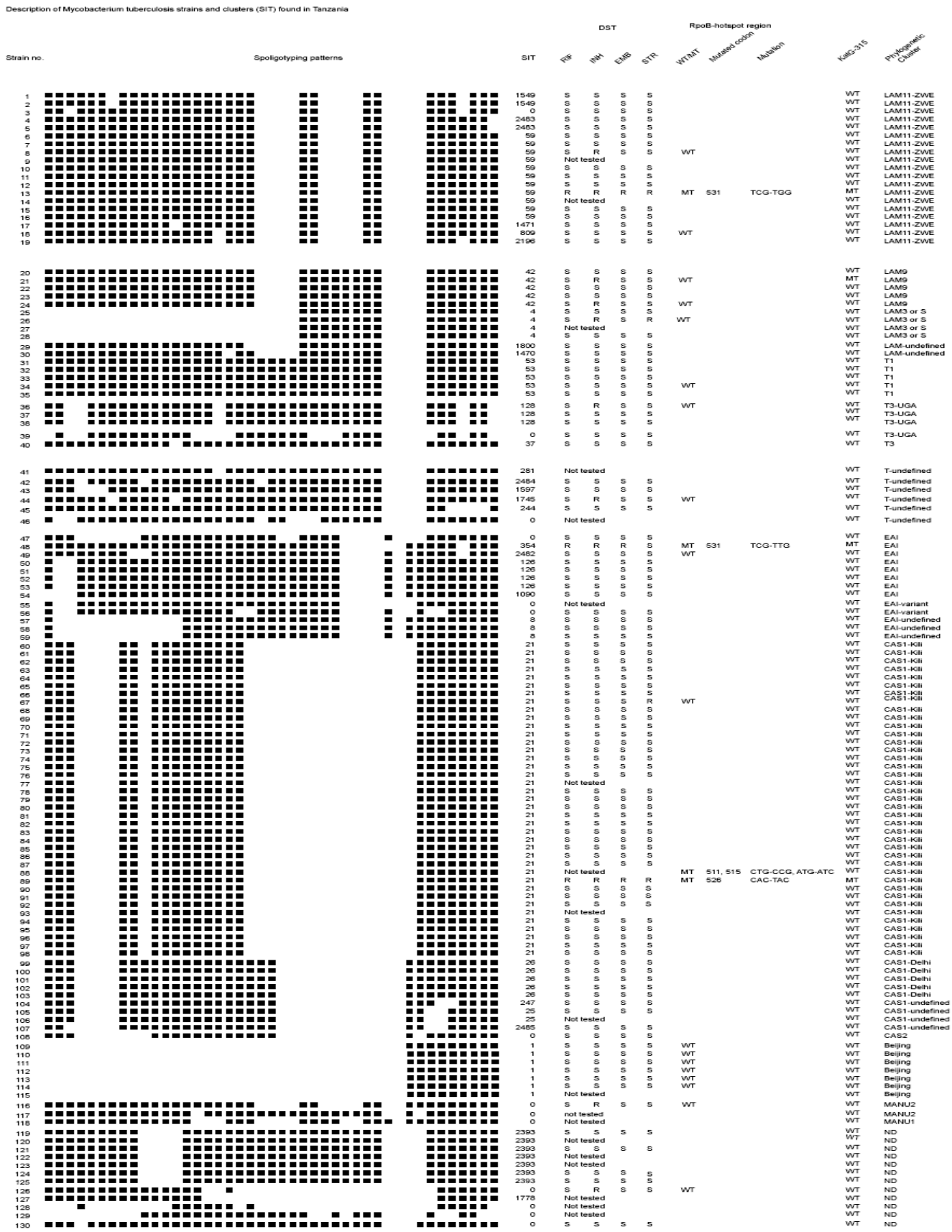

Figure: Description of Mycobacterium tuberculosis strains and clusters (SIT) found in Tanzania

Chapter 7

Serum and BAL macrophage migration inhibitory factor levels in HIV infected Tanzanians with pulmonary tuberculosis or other lung diseases

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ABSTRACT

Background

Macrophage migration inhibitory factor (MIF) activates macrophages, promotes delayed-type hypersensitivity reaction, and regulates Th1/Th2 balance in inflammatory response. Serum MIF concentration is high in patients with pulmonary tuberculosis (PTB). Higher MIF levels are associated with high mortality. No study has addressed MIF levels and its role in PTB/HIV-co infection.

Methods

We determined serum and BAL MIF levels in Tanzanian HIV-infected patients with and without PTB, and correlated the levels with one-month outcome. We compared with serum MIF levels of HIV seronegative patients with PTB and of healthy controls.

Results

All HIV-infected patients irrespective of PTB infection had significantly higher serum MIF levels than HIV- seronegative patients with PTB, and higher than healthy controls. In HIV seropositive patients low serum MIF levels were associated with high one-month mortality.

Conclusion

HIV infection was associated with elevated serum MIF levels regardless of PTB infection. Low serum MIF levels were associated with high mortality.

INTRODUCTION

Macrophage migration inhibitory factor (MIF) activates macrophage functions (e.g. cell adhesion, phagocytosis, and killing of tumour cells and intracellular parasites) (1;2) and localizes macrophages at the inflammatory loci (3). MIF also may have a role of Th1/Th2 balance regulation in inflammatory response and it promotes delayed type-hypersensitivity reaction (4-6). MIF mRNA and protein are constitutively expressed by many organs e.g. brain, pituitary gland, kidneys, liver, spleen, pancreas, skin, and lungs (7-9). In the immune system, MIF has been detected in monocytes, macrophages, dendritic cells, eosinophils, mast cells, basophils, T and B-lymphocytes, and neutrophils (1;9). From these sites, MIF is released after stimulation by microbial products (e.g. bacterial toxins) and after exposure to mycobacteria and proinflammatory cytokines (9;10). In the lungs, MIF is expressed by alveolar macrophages and epithelial cells of the bronchi. Alveolar macrophages play an important role in defense against tubercle bacilli (11).

High MIF concentrations in blood have been found in patients with pulmonary tuberculosis (PTB), bacterial sepsis or shock (8-10). In *M. tuberculosis* infection, virulence of the strain, high concentration of the tubercle bacilli and duration of exposure are associated with strong induction of macrophage MIF production (12). High levels of serum MIF have been found to be associated with severity of tuberculosis, ARDS, sepsis and septic shock (8;10;13;14). Also large quantities of circulating MIF in the body have been associated with increased mortality in diseases caused by both gram-negative and gram-positive bacteria (13).
In animal studies anti-MIF antibody therapy has been shown to prevent toxic shock or death caused by sepsis or bacterial toxins (8;9;14;14-16). This makes

administration of anti-MIF antibodies and MIF antagonists a possible therapeutic option in clinical conditions (3). Therefore, determination of MIF concentration may become important not only as a prognostic index but also as a predictive marker for immunotherapy.

About one-third of the world population is infected with *M. tuberculosis*. By 2000 there were 8.3 million new cases and up to 2.2 million deaths per year due to tuberculosis (17). Human immunodeficiency virus (HIV) infection is a risk factor for tuberculosis. HIV increases the risk of reactivating latent *Mycobacterium tuberculosis* (MTB) (18) and increases the risk of rapid progression of tuberculosis after infection or reinfection with MTB (19). The burden of tuberculosis in countries with a generalized HIV epidemic has therefore increased rapidly over the past decade, especially in the severely affected countries of eastern and southern Africa (20). TB has become the most common disease associated with AIDS and the leading cause of morbidity and mortality (21;22). In these countries up to 31% of the new TB cases are attributed to HIV infection (17). At the same time the diagnosis of PTB and its differentiation from other non-tuberculous pulmonary infections in HIV-infected individuals remains difficult due to poor sputum yield, atypical clinical and radiological presentation.

To our knowledge the clinical significance of MIF levels in serum and in broncho-alveolar lavage (BAL) fluid of PTB/HIV- dual infected patients has not been studied so far. No comparison of MIF levels has been done between HIV seropositive and seronegative individuals in relation to PTB. In this study we determined the concentrations of MIF in serum and BAL fluid of HIV-infected Tanzanian patients with lung disease, some with PTB, and others with lung disease due to other causes. We also determined serum MIF levels in Tanzanian

HIV-seronegative patients with PTB, and in healthy Tanzanian HIV-seronegative volunteers.

MATERIALS AND METHODS

Patients

Between November 2003 and September 2004 we enrolled at Kilimanjaro Christian Medical Centre, a reference hospital for the northern zone of Tanzania, HIV-infected Tanzanian patients with PTB (Group 1: HIV infected/PTB) and without PTB (Group 2: HIV infected/non-tuberculous lung diseases). These two groups were obtained from a larger study (of 120 patients) on chest infections in HIV-infected patients, whereby all patients who qualified for groups 1 and 2 according to the above criteria were enrolled. In addition we enrolled 50 HIV-seronegative individuals as controls: 25 patients with culture-proven PTB (Group 3) and 25 healthy volunteers (Group 4).

Investigations performed in HIV infected patients (groups 1 and 2)

Blood samples were taken for blood cell count, CD4 T cell count and MIF level determination. Bronchoscopy with BAL was performed by standard procedure using flexible fiberoptic bronchoscope (Olympus p45 Tokyo, Japan). Briefly, the bronchoscope was wedged in one of involved segmental bronchi as seen on the chest radiograph. In case of diffuse lung involvement, the scope was wedged in one of the segmental bronchi of the right middle lobe. Then aliquots of 50 ml or less of sterile saline at body temperature was instilled up to a maximum of 150 ml, and sucked back into a sterile container. The BAL fluid was examined for causative microorganisms. Samples for MIF and diagnostic tests were taken prior to the start of anti-tuberculous treatment; however, over 90% of patients

had been on empirical antibiotics for suspected pneumonia before referral. MTB culture was done using Lowenstein-Jensen solid medium after decontamination of BAL fluid with 4% NaOH, centrifugation and taking the sediment. Also, *M. tuberculosis* DNA amplification was performed by real time (rt) PCR of BAL fluid using insertion element IS6110 as a multicopy target for molecular detection of *M. tuberculosis* complex (23). A cut-off cycle-threshold (ct) value of 40 cycles was used. Detection of other organisms (pyogenic bacteria, fungi including *Pneumocystis jiroveci*) was done by staining and culture using standard procedures. In addition the BAL fluid was examined for Human Herpes Virus 8 (HHV8) by PCR. For the HIV-infected patients PTB was diagnosed if both MTB culture and PCR were positive, and non-tuberculous lung disease was diagnosed if both culture and PCR were negative. BAL sediment was stained with haematoxylin & eosin (H&E) for cell analysis to classify inflammation as acute, chronic or subacute based on whether the predominating cells were neutrophils, lymphocytes or both, respectively. Patients were followed up for 4 weeks, when admitted until discharge from hospital, then during clinic visits, or as outpatients for 4 weeks. PTB patients were treated according to Tanzanian guidelines and during the time of the study no antiretroviral treatment was available for HIV infected subjects.

HIV testing was done using Capillus ™ HIV-1/HIV-2 rapid anti-HIV assay (Trinity Biotech, Bray, Ireland) and Vironostika ® HIV uniform II ag/ab microwell enzyme anti-HIV immunoassay (bioMerieux, Marcy 1'Etoile, France). CD4 T cell counting was done by flow-cytometry technique (Becton Dickinson FACS Count machine with BD Facscount™ reagent).

MIF ELISA

The concentration of MIF in BAL (only for the HIV-infected patients) and in serum (for both patients and control groups) was measured by an enzyme-linked immunosorbent assay (ELISA), using the 4-span approach, as described in detail by Grebenschikov et al (24;25). Antibodies were raised in chicken and rabbits using recombinant human MIF as an immunogen. The sandwich structure used included 4 different antibodies, a coating antibody (duck anti-chicken), a capture antibody (chicken anti–human MIF), a trapping antibody (rabbit anti–human MIF), and finally a detection antibody (horseradish peroxidase–labeled goat anti-rabbit). For the standard, human MIF (expressed by *Escherichia coli*) was provided by R&D Systems Europe (Abingdon, UK). The analytic sensitivity for human MIF is 39 pg/ml. Precision profiling showed a coefficient of variation (CV) of 20% at 45 pg/ml (i.e., functional level) that decreased to 7% at higher concentrations. For estimation of the accuracy of this ELISA method, a lyophilized reference preparation (marked 140799) was used. The mean concentration of human MIF in this preparation was 20.7 ng/ml, and the within-run CV and between-run CV amounted to 6% (n = 8) and 12% (n = 11), respectively (over a period of 13 months).

Radiograph

Systematic interpretation of posterior-anterior view chest radiographs was done by a consultant radiologist and reviewed by a chest physician. In case of disagreement a third opinion was sought from another radiologist.

Ethical considerations

Institutional and national ethical clearance was obtained for the study. From all study persons informed written consent was obtained. PTB patients were treated according to Tanzanian guidelines.

Statistics

Using SPSS version 10 statistical package for Windows, normally distributed values were presented as mean with standard deviation (SD). In other cases data were expressed as median with interquartile range (IQR). Spearman's correlation coefficient was used to quantify correlations between continuous variables. Differences between groups were tested with non-parametric Wilcoxon Rank sum test. Linear regression models were used to adjust for potential confounders. P-value equal or less than 0.05 was regarded as statistically significant.

RESULTS

Characteristics of the four study groups

A total of 73 HIV- infected patients with lung diseases were enrolled. In 27 HIV-infected patients, BAL fluid was positive by both culture and PCR for *M. tuberculosis* (Group 1). Age and sex of these patients who were classified as active PTB is given in table 1. In this group median CD4 T cell count was 47.0 (IQR 29.0 – 80.0) cells/μl.

In 46 HIV patients both TB culture and PCR for *M. tuberculosis* in BAL fluid were negative i.e. they had non-tuberculous lung disease (Group 2). In the BAL fluid of these patients, we identified the cause of the lung problem in 22 patients (47.8%): 9 bacterial pneumonia (2 *S. pneumoniae*, 1. *S. pyogenes*, 2 *S. aureus*,

2 *P. aeruginosa*, and 2 coliform bacteria); 5 *Pneumocystis jiroveci*, and 3 fungal infections (*1 Cryptococcus neoformans*, 2 *Aspergillus fumigatus*); 5 HHV8 (indicating possible Kaposi sarcoma of the lung (26)); in the remaining 24 patients (52.2%) no causative organism was identified. Age and sex ratio of these patients is shown in table 1. In this group median CD4 T cell count was 71.5 (IQR 15.0 – 123.8) cells/μl.

Age and sex of the 25 HIV-seronegative PTB cases (i.e. Group 3) and of the 25 HIV-seronegative healthy volunteers (i.e. Group 4) are also presented in table 1**.**

MIF concentration in HIV- infected PTB patients and in HIV-infected patients with non-tuberculous lung disease

Concentration of MIF in the 73 HIV infected individuals ranged from 14.2 to 636 ng/ml in serum (median of 134.2) and from 0.2 to 247.7 ng/ml in BAL (6.9 ng/ml). There was no statistically significant correlation between concentration of MIF in serum and BAL (r^2 = 0.11, p = 0.35).

Median value for serum MIF in the 27 HIV-infected PTB patients was 130.5 ng/ml (IQR 75.5-171.5). Median value for serum MIF in the 46 HIV-infected patients with non-tuberculous lung disease was 136.4 ng/ml (IQR 72.4-213.2). The difference was not statistically significant. Median values for MIF in BAL fluid were much lower than in serum; both in the HIV-infected PTB patients (7.4 ng/ml) and in the HIV-infected patients with non-tuberculous lung disease (5.9 ng/ml) and the difference between the two groups was statistically not significant.

Neither MIF level in serum nor in BAL could discriminate PTB from other lung diseases in HIV-infected patients, even after adjusting for potential confounding factors such as CD4 T cell count, white blood count and age (data not shown)

Correlation of MIF levels with clinical findings in HIV- infected patients.

Serum MIF levels did not correlate with CD4 T cell count (r^2 = 0.18, p = 0.14), white blood cell count (r^2 = 0.34, p = 0.06), neutrophil count (r^2 = 0.06, p = 0.62) or lymphocyte count (r^2 = 0.19, p = 0.11). Similarly BAL MIF levels did not correlate with CD4 T cell count (r^2 = 0.02, p = 0.9), white cell count (r^2 = 0.17, p = 0.14), neutrophil count (r^2 = 0.12, p = 0.33) or lymphocyte count (r^2 = 0.04, p = 0.24). Analysis of H&E stained slides of BAL sediment, showed that concentration of MIF in serum was not associated with acute inflammation when compared with chronic inflammation (r^2 = 0.02, p = 0.9).

In PTB patients, BAL MIF levels were significantly higher in those with nodules on chest radiograph (median 8.82 ng/ml, IQR 2.53 – 19.39), than in patients without nodules (median 2.32, IQR 0.84 – 13.35, Wilcoxon-Rank sum test, p = 0.003). No statistically significant difference was found between median serum MIF levels in PTB patients with nodules on chest radiograph (134.8, IQR 69.5 – 184.8) and in PTB patients without nodules (132.5 ng/ml, IQR 86.6 – 136.0, Wilcoxon-Rank sum test, p = 0.61). No significant differences were found for infiltrates or cavities.

Correlation of MIF levels with clinical outcome

In the 73 HIV seropositive patients (27 with PTB and 46 with non-tuberculous lung disease), 12 patients (16.4%) died due to their lung disease during the one month follow-up period (seven patients with PTB and five patients with non-tuberculous lung disease). The median serum MIF concentration of those who died was 78.8 ng/ml (IQR 26.5 – 139.7) and of those who survived was 138.6 ng/ml (IQR 90.2 – 207.0, Wilcoxon-Rank sum test, p = 0.011). Median BAL MIF concentration of those who died was 9.4 ng/ml (IQR 1.8 – 28.6) and in those

who survived was 5.3 ng/ml (IQR 1.7 – 16.9, Wilcoxon-Rank sum test, p = 0.77). No significant association was found between BAL MIF, CD4 T cell count or WBC (total or differential count) and outcome.

MIF levels in HIV- infected patients, HIV-seronegative patients with PTB and in healthy controls

The median serum MIF concentration in the 73 HIV-infected patients with lung disease (group 1 and 2) was significantly higher (134.2 ng/ml, IQR 75.1 – 190.8) than the serum MIF concentration in the 50 HIV-seronegative individuals (consisting of 25 patients with PTB and 25 healthy volunteers), which was 42.8 ng/ml (33.3 – 70.5, Wilcoxon-Rank sum test, p < 0.0001).

As shown in figure 1, HIV- seropositive patients with PTB had significantly higher median serum MIF levels (130.5 ng/ml, IQR 75.5 – 171.5) than HIV-seronegative patients with PTB (47.6 ng/ml, IQR 34.1 – 152.2, Wilcoxon-Rank sum test, p = 0.004), and higher median serum MIF levels than found in the healthy control group (39.3 ng/ml, IQR 27.0 – 59.3, p < 0.0001).

Interestingly, HIV- seropositive patients with non-tuberculous lung disease had also higher serum MIF concentrations (136.4 ng/ml; IQR 72.4 – 213.2) than HIV-seronegative patients with PTB, (p = 0.016) and healthy volunteers (p < 0.0001; p value not shown in figure 1).

Within the 50 HIV-seronegative individuals, the difference between median serum MIF concentrations (47.6 ng/ml, IQR 34.1 – 152.5) of the PTB group and the healthy volunteers (39.3 ng/ml, IQR 27.0 – 59.0) was also statistically significant (Wilcoxon-Rank sum test, p = 0.03; p value not shown in figure 1).

DISCUSSION

We found high serum MIF levels in HIV-infected patients. The levels were equally high in both, patients with PTB and with non-tuberculous lung disease. The serum MIF levels were about 20-fold higher than BAL MIF levels. No statistically significant correlation in MIF levels was found between serum and BAL in these HIV infected patients. In addition, no statistically significant difference was found in BAL MIF levels between the HIV infected patients with PTB and those without PTB.

Lack of statistical significant differences of serum and BAL MIF levels between patients with pulmonary tuberculosis and those with non-tubercular pulmonary disease shows that HIV infection has a major influence on the levels of MIF and masks the influence of co-infections. Consequently, measuring MIF levels does not help to distinguish patients with PTB, which is a major disease in HIV, from those patients with non-tuberculous pulmonary diseases.

We found no statistically significant differences in serum MIF levels among different chest radiographic patterns. A study in non-HIV patients with PTB similarly showed no significant differences of serum MIF levels among radiological stage groups (10). However in our study we found that presence of nodular lesions in the chest radiographs of patients with PTB correlated with high levels of MIF in BAL fluid. This suggests possible correlation between MIF levels in the lungs and radiographic stages in PTB/HIV co-infected patients.

Lower levels of circulating MIF in HIV infection were associated with increased mortality at one-month follow-up. This finding differs from findings in other studies in animals and human beings (8;10;13). In these studies higher levels of

circulating MIF were clearly indicative of severe disease and fatal outcome. This finding in our study therefore raises doubts on the role of anti-MIF therapy in lung diseases in HIV-infected individuals.

In TB/HIV dual infection, HIV infection has been found to facilitate fast progression of TB (19) and TB to enhance replication and progression of HIV infection (27;28). The interaction of the dual infection can result into altered cytokine regulation (11). We postulate that the results in this study may be influenced by such alteration.

In this study, serum MIF levels were significantly higher in patients with PTB/HIV co-infection than in patients with PTB without HIV infection; pointing to the influence of HIV infection on high MIF levels. And also the serum MIF levels of PTB/HIV co-infected patients in this study were nearly 10-fold higher than MIF levels which were found by *Yamada et al* in PTB patients without HIV infection (10).

Furthermore, we also observed that our PTB patients without HIV infection had higher MIF levels than that of a similar group of patients (i.e. PTB without HIV-infection) in the Japanese study (10). The median value in our study was more than two-fold higher than the mean serum MIF concentration in the Japanese study.

Similarly, the Tanzanian healthy HIV seronegative control group had nine-fold higher levels of MIF than that of the healthy HIV seronegative control group in the study by Yamada *et al* (10), which had a mean of 4.38 $\pm$ 1.34 ng/ml. And

also the serum MIF levels in the Tanzanian volunteers were three-fold higher than the levels measured in healthy Dutch volunteers (median/IQR = 13.2 /10.0 – 16.5).

Apart from the differences in immunoassays (e.g. antibody characteristics and calibration standards), the differences between different study populations may be due to factors such as environmental or genetic in the human host. Factors such as MIF polymorphism, subclinical, non-symptomatic condition or chronic infection (e.g. latent tuberculosis, which is present in the majority of healthy adult Tanzanians) may have influenced the comparatively higher MIF levels of the HIV seronegative Tanzanians. The differences we have found between control groups emphasize on the importance of selecting an appropriate control group, which genetically and environmentally is closely linked to the study population.

Although the levels of serum MIF in the healthy Tanzanian volunteers in this study, was comparatively higher than healthy control groups in other studies, the levels were still statistically significantly lower than MIF levels of the 25 HIV seronegative Tanzanians with PTB.

In summary, in this study among Tanzanian subjects, HIV infection appeared to be associated with elevated serum MIF levels. The MIF levels could not discriminate PTB, a major disease in HIV infection from other lung diseases. The effect of PTB on MIF levels in HIV co-infection was masked by HIV infection. Low levels of MIF were associated with increased one-month mortality. This finding

indicates that further safety studies are warranted before immunotherapy using anti-MIF antibodies can be applied as part of treatment.

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Table 1. Characteristics of the study populations

Group 1: HIV - infected patients with PTB (n = 27)	
Age, mean (SD)	39.2 (8.9)
M: F ratio	1: 1.1
CD4 count, median (IQR)	47.0 (29.0 - 80.0)
Group 2: HIV-infected patients with non-tuberculous lung disease (n = 46)	
Age, mean (SD)	39.3 (10.6)
M: F ratio	1: 1.2
CD4 count, median (IQR)	71.5 (15.0 - 123.8)
Group 3: HIV-seronegative patients with PTB (n = 25)	
Age, mean (SD)	33.2 (13.8)
M: F	2.6: 1
Group 4: HIV-seronegative healthy volunteers (n = 25)	
Age, mean (SD)	38.7 (10.9)
M: F ratio	1: 1.1

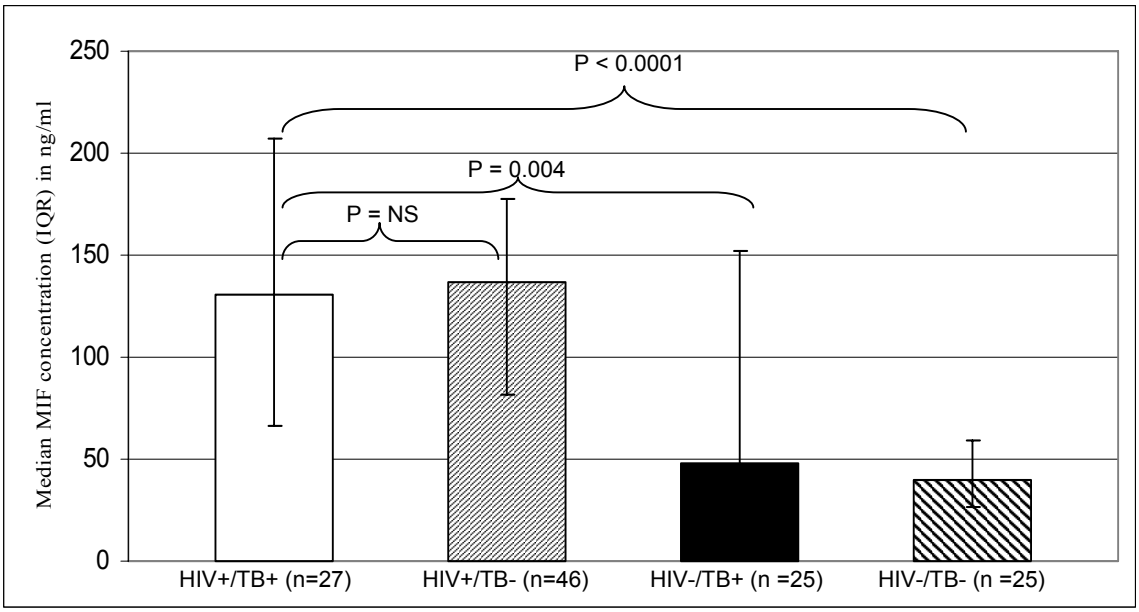

Figure 1. Median serum MIF and IQR in the four groups: HIV-seropositive with and without PTB and HIV-seronegative with and without PTB. The difference between serum MIF in the HIV-infected patients with PTB or without PTB was not statistically significant. Serum MIF in HIV- infected patients with PTB was higher than in HIV-seronegative with PTB ($p = 0.004$) as well as without PTB ($p < 0.0001$).

Chapter 8

Bronchoalveolar neutrophils, IP-10, and IL-7 in AIDS-associated tuberculosis

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ABSTRACT

Background

During advanced AIDS tuberculosis often presents atypically with smear-negative and non-cavitary disease, yet immune features associated with this change are poorly characterized.

Methods

We examined the local immune response in a cohort of Tanzanian AIDS-associated tuberculosis patients that underwent bronchoalveolar lavage. Tuberculosis infection was confirmed in BAL fluid by culture, probe, and PCR.

Results

Among tuberculosis patients CD4 count correlated positively with the extent of cavitary disease as well as BAL Tb load (qPCR C_T). Tb patients had significantly higher GM-CSF than non-Tb patients, and those with non-cavitary Tb had significantly higher BAL IP-10 and IL-7 than those with cavities. BAL neutrophils were as prevalent as monocytes/macrophages or epithelial cells, and immunohistochemistry revealed that neutrophils, monocytes/macrophages, and epithelial cells were major sources of the IP-10 and IL-7.

Conclusion

These data suggest a dysregulated cytokine profile may contribute to the Tb of advanced AIDS.

INTRODUCTION

Mycobacterium tuberculosis (Tb) is the world's most deadly bacterial pathogen [1]. Tb rates and mortality are higher in HIV infected persons[2] and Tb accelerates HIV replication and heterogeneity [3, 4]. The tendency for tuberculosis to present atypically during AIDS, without the hallmark of upper lobe cavitary lung disease, is well-established [5-8]. A proposed explanation for the lack of lung cavitation with advanced AIDS is the loss of a CD4-mediated IFN-γ or DTH response to drive the granuloma and cavitation process [9, 10]. Unfortunately, supporting data that evaluate the cytokine profile from AIDS-associated Tb patients are few, particularly from the bronchoalveolar compartment, and particularly from sub-Saharan Africa where disease burden is highest. We therefore sought to examine the immune characteristics of AIDS patients with well-documented Tb infection across a range of CD4 counts and radiographic presentations.

MATERIALS AND METHODS

Patient population and bronchoscopy

Informed consent was obtained from all participants and the University of Virginia Human Investigation Committee and the Kilimanjaro Christian Medical Centre Ethics Committee reviewed and approved the project. Bronchoscopy with bronchoalveolar lavage was performed by standard procedure using a flexible fiberoptic bronchoscope (Olympus p45, Tokyo, Japan) wedged into involved segmental bronchi. In the case of diffuse lung involvement, the scope was wedged into one of the segmental bronchi of the right middle lobe.

Detection of Tuberculosis

Each BAL fluid was assayed by AFB smear (at KCMC), culture (2 ml on solid and liquid media at UVA), PCR (at UVA, see below), and by Ziehl-Neelsen stain of BAL cell block (at UVA, see below). Tuberculosis infection was defined by positive culture with confirmation of *M. tb* complex by DNA probe (n = 13). The Tb-negative group (n = 21) was defined as AFB smear, culture, and PCR negativity. Eight patients were excluded from further analysis because their Tb status was uncertain (5 were smear positive but culture negative and PCR negative; 3 were smear negative and culture negative but PCR positive). Four patients were excluded because their BAL grew non-tuberculous mycobacteria and we could not rule out overgrowth of Tb. Among the non-tuberculosis cases, 10 had the bronchoscopic appearance Kaposi's sarcoma. Other diagnostic tests were performed per the attending physician's orders but were not revealing.

Tb PCR

Real time PCR for the IS6110 gene was performed on BAL fluid using the assay and primers of Pounder et al [11]. Briefly, 2 ml of the BAL fluid was centrifuged at 16,000 × *g* for 5 min and 200 μl of PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA) was added to the pellet. The mixture was vortexed, boiled for 15 min, and centrifuged at 16,000 × *g* for 5 min to remove cellular debris. PCR of 5 μl of extracted DNA was performed in a total volume of 25 μl containing 12.5 μl of iQ SYBR Green Supermix 2× (Bio-Rad, Hercules CA), iTaq DNA polymerase (50 U/ml), 6 mM $MgCl_2$, 20 nM SYBR Green I, 0.5 μM of each primer, 100 ng *M. tb* DNA for positive control, and water for negative control. Cycling conditions entailed 1 cycle at 95°C for 15 min followed by 40 cycles at 95°C for 10 s, 58°C for 20 s, and 76°C for 20 s.

Radiographic analysis

All patients had chest radiographs read in blinded fashion by a radiologist (C.F.K.). CXR scores were determined as previously described [12]. The extent of cavitation was estimated by measuring the diameter of each cavity (mm) on CXR, assuming cavities were roughly spherical ($4/3\ \pi\ r^3$), and summing the volumes of all cavities.

CD4T cell count

CD4 T cell count was measured using the Coulter Manual CD4 Count kit (Beckman Coulter, Hialeah, FL).

Cytokine measurements.

Five ml of BAL fluid was added to DTT (0.05% final concentration) to dissolve mucus and filtered with 0.22 μm filters (MILLEX GP, Millipore, Cork, Ireland). Fluid was then concentrated in Amicon Ultra -15 filters (10,000 MWCO; Millipore) and assayed for 22 cytokines/chemokines using the human 22-plex cytokine kit (Upstate, Charlottesville, VA) on a Luminex-100 instrument according to the manufacturer's instructions. For TGF-β1 quantitation, samples were acid-activated to pH 1.5 with 1N HCl for 60 minutes prior to assay. To adjust for BAL dilution, all cytokine data was normalized to the total protein content in the fluid as determined by BCA assay (Pierce, Rockford, IL).

Histology and immunohistochemistry (IHC)

Ten ml of BAL fluid was stored in RNALater (Ambion, Austin, TX) at –70°C and cell blocks were made by pelleting the material, fixing in 10% formalin, and embedding in paraffin. Tissue sections were stained with hematoxylin and eosin,

Ziehl-Neelsen, and IHC was performed for IP-10 and IL-7. All pathology was scored blindly by a pathologist (L.M.). For each sample a cellularity score was determined for epithelial cells, monocytes/macrophages, lymphocytes, and neutrophils as follows: 0 = none, 1 = few, 2 = scattered, 3 = many. Ziehl-Neelsen stained cell blocks were viewed at 1000× and scored for AFB positivity as follows: 0 = none, 1 = rare, 2 = few, 3 = scattered throughout. For IHC, 3 micron-thick paraffin sections were obtained using a Leica Microtome, mounted on superfrost/PLUS glass slides and deparaffinized to deionized water. Samples were incubated overnight at 4°C using IP-10 and IL-7 antibodies (R & D systems, Minneapolis, MN) with dilutions ranging from 5 μg/ml to 125μg/ml. Samples were rinsed in PBS and incubated with a biotinylated secondary antibody for 30 minutes at room temperature. Samples were then incubated with the ABC kit (Vector, Burlingame, CA) for 30 min at room temperature, rinsed in PBS, and stained with DAB Substrate (Vector) before counterstaining. An IHC score was determined for each cell population (epithelial cells, monocytes/macrophages, lymphocytes, and neutrophils) according to the following scale: 0 = weak, majority of cells not staining; 1 = intermediate; 2 = strong, majority of cells staining.

Statistics

Means and medians were compared using t test and Mann-Whitney test, respectively. Correlations were measured using the Pearson linear correlation. All *P* values are two-tailed. Data shown are mean ± SD unless otherwise indicated.

RESULTS

Utility of BAL PCR for detection of Tuberculosis infection

The endoscopy unit at the Kilimanjaro Christian Medical Centre is one of few centers in the region equipped for bronchoscopy and serves as a referral center for patients with chest disease of unknown etiology. We enrolled and consented 46 such patients known to be HIV-positive and performed Tb diagnostics and cytokine analysis on BAL. Tuberculosis infection was identified in 13/46 (28%) by culture with confirmation of *M. tb* complex by DNA probe. Against this gold-standard, BAL PCR exhibited better sensitivity and specificity than BAL AFB smear (100%/91% vs. 54%/85%). Furthermore real time PCR C_T (cycle threshold) exhibited the expected quantitative correlation with time to positive culture (C_T vs. days to positivity; r = 0.68, *P* = 0.01) and AFB score (C_T vs. AFB score; r = 0.67, *P* = 0.02).

CD4 count correlates with cavitary disease and endobronchial Tb burden

There were no significant differences between the Tb-positive and Tb-negative populations in terms of age, gender, CXR score (4.8 ± 1.4 vs. 7.4 ± 0.9, *P* = NS), or CD4 count (194 ± 41 vs. 146 ± 31, *P* = NS). Among tuberculosis patients, average CD4 count was higher in patients with cavities as detected by CXR (Fig. 1A). Additionally, CD4 count correlated positively with cavitary volume and BAL Tb burden (qPCR C_T; Fig 1B, C).

BAL cytokine/chemokine response in AIDS-associated tuberculosis

Scant data is available on the BAL response during AIDS-associated Tb. Most reports derive from HIV-negative Tb in developed countries and findings have been extremely variable [13-15]. First, we examined the cell blocks prepared

from the BAL fluid. As expected, lymphocytes were scant (cellularity score 0.6 ± 0.5), but we were surprised to find a substantial amount of neutrophils, many of which contained intracellular *M. tuberculosis* bacilli (Fig. 2; cellularity scores 2.1 ± 0.9 for neutrophils, 2.3 ± 0.7 for monocytes/macrophages, and 2.0 ± 0.6 for epithelial cells, respectively, *P* = NS). We next performed a broad characterization of 23 chemokines and cytokines in BAL fluid using the Luminex assay, with all cytokine data normalized for dilution against the total protein content in the fluid. The analysis showed that the Tb-positive group exhibited higher BAL GM-CSF levels than Tb-negative individuals (the definition of tuberculosis-negativity required AFB smear, culture, and PCR negativity; *P* = 0.007), while all other cytokines/chemokines were not statistically changed (Table 1). Upon comparing the immune profile within the Tb group, individuals with non-cavitary Tb had significantly higher BAL IL-7 and IP-10 than those with cavitary disease (*P* = 0.02). Other cytokines/chemokines were not significantly associated with radiographic findings or CD4 count. Finally, immunohistochemistry of BAL cell blocks revealed that IP-10 was produced by epithelial cells, monocytes/macrophages, and neutrophils but not lymphocytes (IHC positivity score 1.5 ± 0.5, 1.6 ± 0.5, 1.2 ± 0.8, 0.1 ± 0.4, respectively; *n* = 12, *P* = NS between epithelial cells, monocytes/macrophages, and neutrophils). Meanwhile IL-7 was produced predominantly by epithelial cells and monocytes/macrophages (IHC score 1.5 ± 0.7, 1.3 ± 0.6, versus 0.7 ± 0.6 and 0.0 ± 0.0 for neutrophils and lymphocytes; *n* = 11, *P* < 0.05 comparing epithelial cells and monocytes/macrophages versus neutrophils or lymphocytes). For both IP-10 and IL-7, the absence of lymphocyte staining may certainly reflect their paucity given HIV infection.

DISCUSSION

The importance of this work lies in the characterization of the immune profile at the site of infection from a substantial number of HIV-infected, probe-confirmed Tb patients from sub-Saharan Africa. Many studies from developing world sites perform only smear or culture but not probe or PCR for confirmation of *M. tb* complex. This would have led to a significant rate of erroneous assignment to both the Tb-positive and Tb-negative groups in our population. As a noteworthy aside, we feel that PCR directly on BAL, while technically demanding in a resource limited setting, has tremendous advantages in terms of accuracy over smear and rapidity over culture/probe.

The comparison of cytokine responses between the Tb and non-Tb groups revealed a statistical increase in BAL GM-CSF in the setting of Tb, a finding that has not been previously reported. One might predict this chemokine may exert a partially protective response given its role in Tb containment and granuloma development in mice [16]. Whether additional exogenous GM-CSF would be therapeutic in active Tb is unknown: in one small study clinical improvement was modest [17]. Potentially driven by the increase in GM-CSF, we were struck by the BAL neutrophilic response we saw in these Tb patients, particularly given the abundant bacilli within the neutrophils. The role of neutrophils in Tb is unresolved: intracellular killing has been demonstrated in vitro by some but not others [18, 19]. In mouse models, neutrophils do not affect survival but do contribute to granuloma formation [20, 21]. Taken together, we speculate that the increased GM-CSF and neutrophil response is an attempt by the host to control Tb, particularly in the setting of AIDS.

The association we found between CD4 count and cavitary disease and endobronchial Tb burden was not surprising. Several studies have documented this trend [5-8, 10], supporting the hypothesis that CD4 cells contribute to the pathogenesis of cavitary disease and endobronchial spread. The mechanism by which this cavitation process occurs is clearly of paramount importance to the AIDS/Tb epidemic due to its implication on Tb transmission. We therefore sought to examine the cytokine/chemokine profile between the cavitary and non-cavitary groups. Based on limited human or mouse data we predicted a correlation between loss of cavitation and a loss of TNF-α or IFN-γ [22-24], however diminution of these cytokines in the non-cavitary group was modest and not statistically significant. More readily apparent was the association between the loss of cavitation and IL-7 and IP-10. To our knowledge IL-7 has not been reported in the BAL of Tb patients. One could postulate that the IL-7 is merely a marker for the advanced AIDS of the non-cavitary group given its association with CD4 decline [25], however IL-7 may be relevant to HIV pathogenesis via its upregulation of RANTES (and thereby promotion X4 virus) [26]. The localization of IL-7 production by immunohistochemistry to bronchial epithelial cells and macrophages was not surprising and has been reported by others [27, 28].

IP-10 is an IFN-γ inducible chemokine critical for effector T cell development and trafficking in mice [29]. Its elevation during tuberculosis has been reported [20, 30], however a neutrophilic source has not been described. Some authors postulate a protective role for this chemokine [31] yet our findings of extremely high levels in the advanced AIDS/non-cavitary group would argue against an unequivocally protective function for IP-10. Indeed given the positive effect of

IFN-γ on IP-10 transcription (as well as that of IL-1α and TNFα in some systems [32]), the high IP-10/low IFN-γ/low IL-1α/low TNFα pattern of the non-cavitary group was unexpected and suggest loss of IP-10 regulation or induction by other known mediators such as TLR ligands [33, 34].

Human data are important to direct research in experimental and animal models, particularly given the complexity of AIDS-associated Tb which cannot be easily replicated. Based on our findings we question the role of GM-CSF and neutrophils during AIDS-associated Tb. Additionally, the function of IL-7 on Tb-specific HIV progression and of IP-10 in T cell trafficking in tuberculosis would seem fertile topics for research.

Acknowledgments

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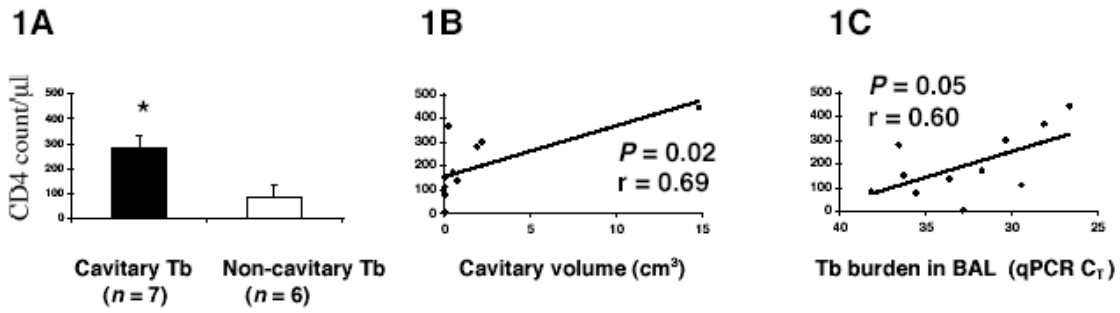

Figure 1. Association between CD4 count, cavitary disease, and Tb PCR in BAL. CD4 count of HIV+/Tb+ patients was compared (A) between those with and without cavitary findings on CXR, (B) versus total cavitary volume, and (C) versus BAL PCR cycle threshold (C_T). Figure A shows mean + SE; *, $P < 0.05$ by t-test or Mann-Whitney test. Correlations used the Pearson linear correlation.

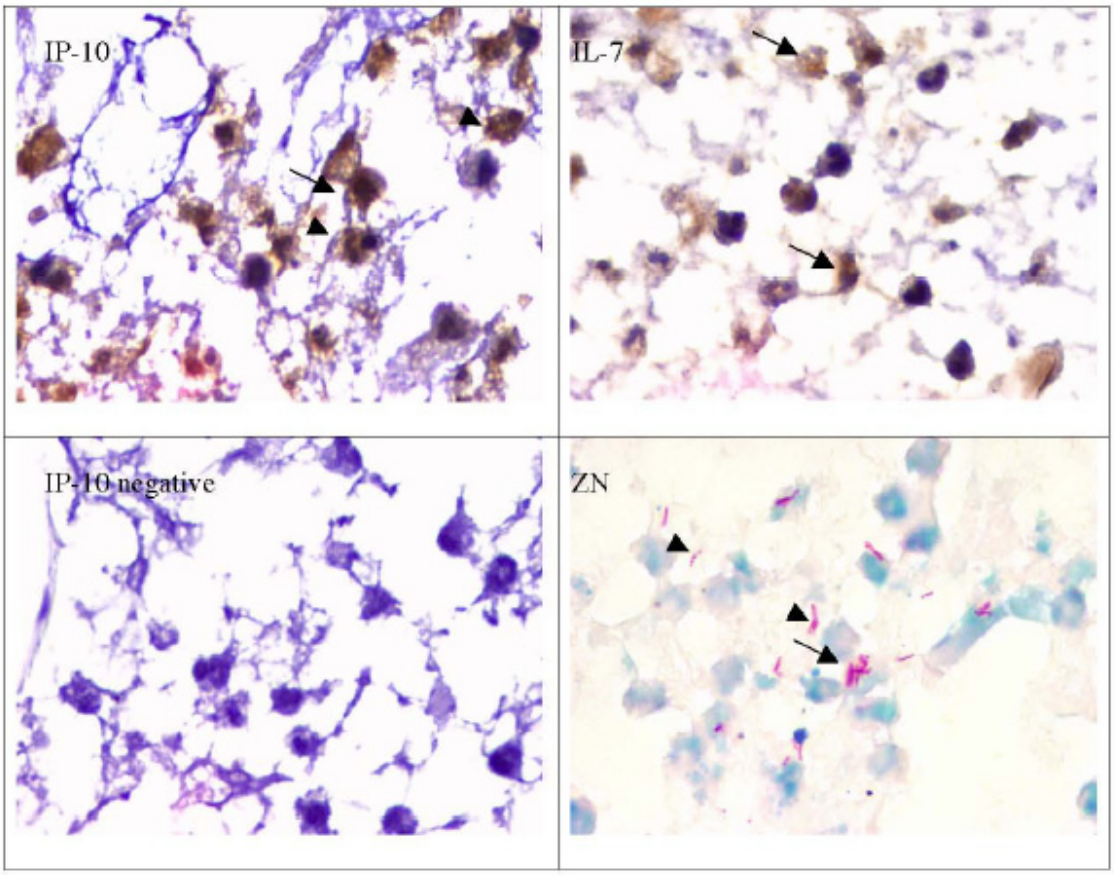

Figure 2. IP-10 and IL-7 in AIDS-associated tuberculosis. Formalin-fixed, paraffin-embedded cell blocks were prepared from BAL fluid and immunohistochemistry performed with anti-IP-10 and anti-IL-7 primary antibody. Positive staining macrophages (arrows) and neutrophils (arrowheads) are shown. No background staining was observed without IP-10 (lower left) or IL-7 antibody (not shown). Ziehl-Neelsen (ZN) stain shows *M. tuberculosis* bacilli both inside neutrophils (arrowhead) and extracellularly (arrow). All photomicrographs come from one of the seven patients with non-cavitary tuberculosis.

TABLE 1. Comparison of BAL cytokine/chemokines between HIV-infected patients with cavitary Tb, non-cavitary Tb, and without tuberculosis

Cytokine/ chemokine	Tb cavitary (*n* = 6)	Tb non-cavitary (*n* = 7)	*P* (cavitary vs. non-cavitary)	Non-Tb (*n* = 21)	*P* (Tb vs. non-Tb)
IL-1α	205.3 ±	112.9 ± 43.4	NS [a]	143.3 ± 33.1	NS
IL-1β	91.8 ±	116.1 ± 52.7	NS	93.0 ± 24.6	NS
IL-2	6.3 ± 1.5	4.6 ± 1.4	NS	5.7 ± 0.9	NS
IL-3	1.1 ± 1.1	ND[b]	NS	1.9 ± 1.2	NS
IL-4	3.7 ± 1.2	2.9 ± 1.2	NS	2.6 ± 0.6	NS
IL-5	10.3 ± 2.3	10.7 ± 1.7	NS	8.8 ± 1.0	NS
IL-6	73.0 ±	306.9 ± 94.3	NS	136.7 ± 43.0	NS
IL-7	28.0 ± 5.9	57.5 ± 7.3	0.02	32.0 ± 3.7	NS
IL-8	456.5 ±	1272.2 ±	NS	730.4 ±	NS
IL-10	ND[b]	5.5 ± 4.5	NS	1.1 ± 0.6	NS
IL-12p40	47.3 ±	61.2 ± 14.3	NS	47.3 ± 14.1	NS
IL-12p70	8.0 ± 1.8	12.9 ± 2.8	NS	10.3 ± 1.3	NS
IL-13	8.1 ± 1.6	10.2 ± 1.8	NS	8.7 ± 1.1	NS
IL-15	4.8 ± 2.2	7.9 ± 3.8	NS	7.5 ± 1.4	NS
IFN-γ	354.7 ±	59.9 ± 16.2	NS	63.8 ± 12.8	NS
TGF-β1	2.2 ± 1.1[c]	2.8 ± 1.5	NS	1.7 ± 0.5 [c]	NS
TNF-α	25.6 ±	10.3 ± 5.9	NS	10.3 ± 3.5	NS
EOTAXIN	54.6 ±	102.1 ± 27.0	NS	82.0 ± 11.9	NS
GM-CSF	8.1 ± 1.6	12.9 ± 2.2	NS	5.1 ± 1.0	0.007
IP-10	350.5 ±	1709.9 ±	0.02	306.2 ± 75.1	NS
MCP-1	42.6 ±	117.6 ± 39.7	NS	47.3 ± 6.2	NS
MIP-1α	56.8 ±	57.9 ± 17.5	NS	46.7 ± 5.0	NS
RANTES	37.0 ± 4.7	64.2 ± 14.8	NS	318 ± 3.2	NS

Data shown as mean ± SE pg cytokine/mg protein.

[a] NS = not statistically significant. *P* values reflect t-test, however all significant *P* values were also met when comparing medians (Mann-Whitney test).

[b] ND = not detected for any specimen.

[c] TGF-β1 data available for only 6 cavitary Tb specimens and 19 non-Tb specimens.

Chapter 9

The toll-like receptor 4 Asp299Gly variant and tuberculosis susceptibility in HIV-infected patients in Tanzania

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ABSTRACT

Background

Toll-like receptor 4 (TLR4) plays an important role in the pattern-recognition of *Mycobacterium tuberculosis*, and polymorphisms in the TLR4 gene influence the function of the receptor. We therefore investigated in a cohort of HIV-infected Tanzanian patients whether the Asp299Gly TLR4 polymorphism is associated with the development of active tuberculosis. We found a higher risk of developing active tuberculosis as well as a reduction in CD4 T cell counts in patients with Asp299Gly polymorphism.

INTRODUCTION

Due to the growing number of HIV infections in sub-Saharan Africa, the number of co-infections of HIV and *Mycobacterium tuberculosis* is also increasing fast, with a significant contribution to morbidity and mortality (1;2). It is well known that the incidence of infection with *M. tuberculosis* is much higher than that of active tuberculosis (TB), even in the case of co-infection with HIV. Toll-like receptors are a class of receptors which are important for the recognition of pathogens by the innate immune system and for bridging the innate and acquired immune response (3). Research revealed that TLR4 mediates cytokine production stimulated by *M. tuberculosis*, and that TLR4 knock out mice are more susceptible to infection with *M. tuberculosis* (4;5). Therefore we wanted to asses whether the TLR4 polymorphism Asp299Gly, which is common in Africa, is involved in the development of active TB in HIV infected patients.

MATERIALS AND METHODS

At Kilimanjaro Christian Medical Center (KCMC) in Tanzania, we enrolled 120 HIV infected patients presenting with symptoms of chest infection and/or chest radiographic abnormalities. These patients underwent bronchoscopy with collection of bronchoalveolar lavage (BAL) fluid for TB culture and real time PCR (qPCR) DNA amplification for *M. tuberculosis* (6). These results were used to form: Group 1; Active TB disease (culture and qPCR positive) and Group 2; no active TB (culture negative and qPCR negative or positive). Patients with a negative culture and positive qPCR were followed during 18

months whereby none developed active TB. Patients on anti-TB treatment were regarded as active TB cases if qPCR was positive despite negative culture. Sixteen patients were excluded because of incomplete data, thus a total of 104 patients remained for analysis. From all these patients the TLR4 Asp299Gly polymorphisms was determined, as described before (7).

RESULTS

Twenty-four patients had active TB and 80 had no active TB. Gender and age did not differ between the two groups. The percentage of Asp299Gly polymorphism of patients with no active TB was 7.5%, (N = 6 /80) compared to a 2.8-fold higher prevalence within the active TB group (20.8%, N = 5/24). This difference almost reached significance (χ^2 P = 0.06), suggesting that HIV-infected patients carrying the Asp299Gly allele have an increased susceptibility to develop active TB. These findings are in line with the in-vivo reports that TLR4 is a receptor involved in the recognition of the soluble factor of M*. tuberculosis* and for whole mycobacterium ligands (8;9). Difference between our results and those of Newport *et al*, who was not able to find a role of TLR4 Asp299Gly polymorphism in non-HIV infected individuals, indicates that the role of TLR4 recognition and activation of the innate immune system is likely to be more pronounced in HIV infected individuals (10-12). Another cause of the differences between the studies may be the different genetic background (West-Africa / Gambia and East-Africa / Tanzania), as the ethnicity is known to interact with TB susceptibility (12;13).

Because all patients were HIV-infected, we also looked at the difference between CD4 T cell counts. As expected, patients with active TB had a lower median CD4 T cell count compared with the no active TB group (Mann-Whitney U-test, P = 0.01, Figure 1A). Interestingly, within the group of patients without the Asp299Gly polymorphism, median CD4 T cell counts were significantly lower in individuals with active TB, compared to individuals in the no active TB group (Mann-Whitney U-test, P = 0.01, **Figure 1A**). This difference was no longer present in patients with the Asp299Gly polymorphism who all except one had CD4 T cell counts below 100 cells/mm^3 (Mann-Whitney U-test, P = 0.79) (Figure 1A). Further investigation revealed that distribution of the Asp299Gly polymorphism in subgroups of patients according to the clinically-important categories of CD4 T cell counts (350, 200, 100 cells/mm^3, **Figure 1B**) showed increased prevalence of the Asp299Gly polymorphism in all subgroups of patients having CD4 T cell counts below 350, 200 and 100 cells/mm^3. However, only in patients with CD4 T cell count below 100 cells/mm^3 the prevalence of the Asp299Gly polymorphism (15.6%) was significantly higher than in patients with CD4 T cells of more than 100 (2.5%; χ^2 P = 0.03). It should be noted that only one individual with the Asp299Gly polymorphism had CD4 T cell counts above 100 (Figure 1B). On the one hand, this striking observation is likely due to the mechanisms through which Asp299Gly increases susceptibility to TB. On the other hand, this phenomenon is likely to be important for the pathology and clinical presentation of HIV infection because both therapeutic and antibiotic prophylaxis decisions are based on CD4 T cell counts. So the

surprising difference between CD4 T cell counts within TLR4 polymorphisms indicates that the HIV-infected status is important for the impact of TLR4 Asp299Gly. CD4 T cell counts are the most important prognostic factor in HIV-infection for the development of opportunistic infections, including TB.

DISCUSSION

Different factors may explain the observed low number of CD4 T cells in HIV-infected patients bearing the TLR4 Asp299Gly polymorphism. TLR4 is known to activate the interferon response, thus inhibiting HIV replication. Therefore, individuals bearing the TLR4 Asp299Gly polymorphism may have a more pronounced viral replication, with enhanced loss of CD4 T cells and increased susceptibility for TB. Another explanation can be that the absence of potent inflammation in TLR4 Asp299Gly HIV-infected individuals leads to less symptoms due to infection with *M. tuberculosis*, therefore patients will present to the hospital at a later stage of the disease. Additional studies with prospective follow-up of newly diagnosed HIV-infected patients bearing either wild-type or the Asp299Gly allele is needed to answer these questions.

In conclusion, Asp299Gly polymorphism at the level of TLR4 is associated with an increased chance to develop active TB in HIV-infected patients, and this seems to involve an effect of this polymorphism on the CD4 T cell counts in these patients.

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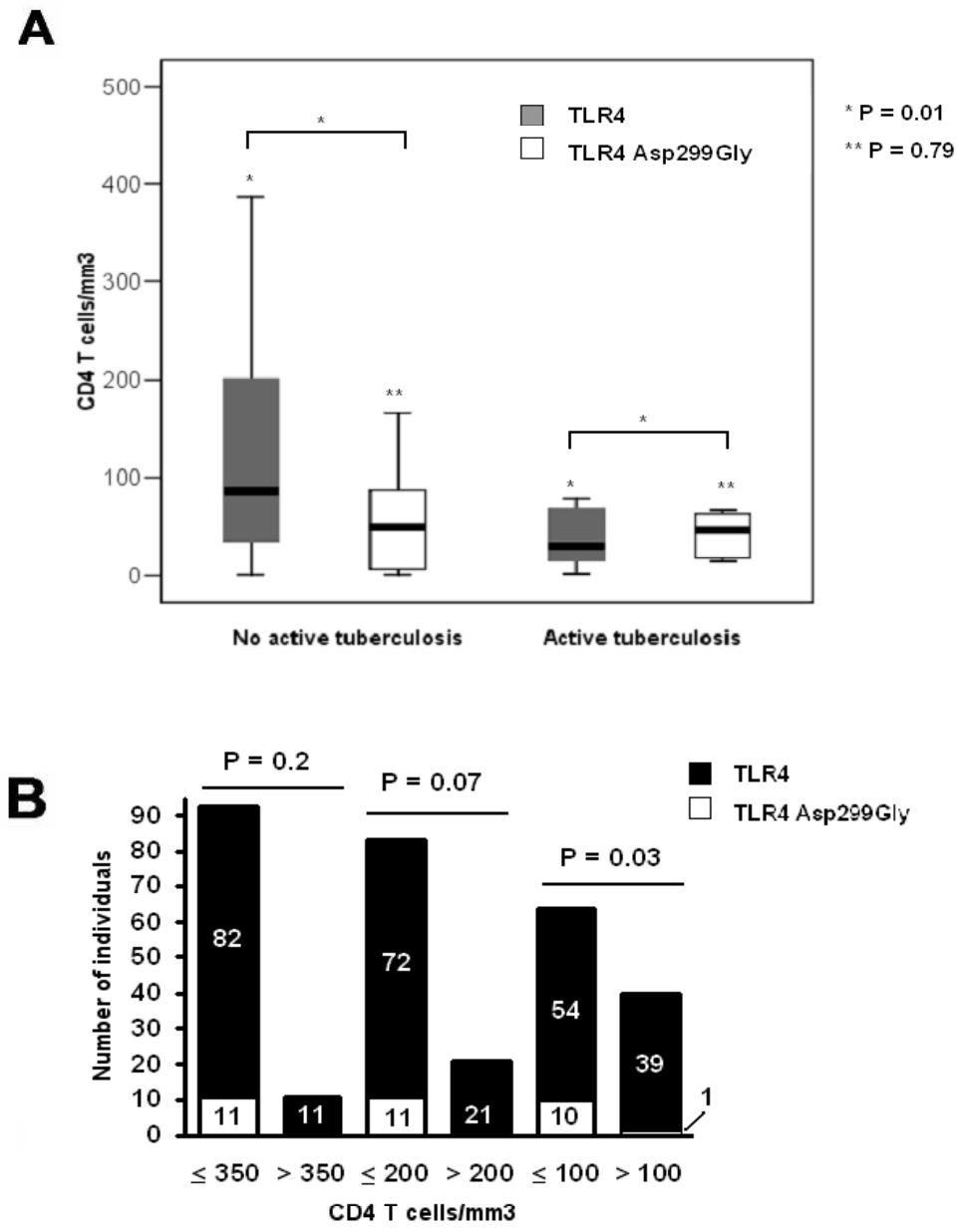

Figure 1: (A) Median CD4 T cell counts of the 104 patients studied grouped by their tuberculosis and TLR4 polymorphism status. **(B)** Distribution of the 299 allele of the patients when they were grouped by CD4 T cell counts equally to or above, and below 350, 200 and 100 cells/mm^3, respectively. Numbers in the bars represent numbers of patients in each.

Chapter 10

General discussion and avenues for future research

GENERAL DISCUSSION

This thesis centres on clinical and immunological studies of tuberculosis (TB) in patients with HIV infection. It comprises a range of studies, firstly clinical studies on the impact of HIV infection on morbidity and mortality in a referral hospital. Next, diagnostic studies on the aetiology of chest problems in HIV infected patients, diagnostic problems in TB in HIV infection and on the role of human herpes viruses (HHV) as co-infection. Then follows a study on *M. tuberculosis* (MTB) genotypes and TB drug resistance. Finally, several pathophysiological studies are presented comprising immunological responses of the host to MTB in a HIV milieu and a role of TLR4 polymorphism in the development of active TB. These studies were done in Tanzania, one of the sub-Saharan countries heavily affected by the two diseases (1-5). Data on TB and HIV infection from sub-Saharan Africa are sparse and insufficient.

Our quest to research more on co-infection between TB and HIV was triggered by the findings of our initial study **(chapter 2)** which showed that pulmonary infections were the leading causes of hospitalization, morbidity and in-hospital mortality, and MTB was the leading single infectious cause of morbidity and mortality, a finding which is similar to other studies (2;6). The impact was made worse by a low clinical suspicion index for HIV infection. We found that only about a half of the actual HIV infected in-patients were tested based on clinical suspicion that HIV infection was the underlying disease. The two infections (TB and HIV) in this way amount to serious

negative socio-economical consequences for this economically already constrained country, as well as to a heavy burden on the health care system.

The apparent role of pulmonary infections in morbidity and in-hospital mortality in HIV infected individuals observed, prompted a study into the causes of these infections **(chapter 3),** where we used bronchoalveolar lavage (BAL) fluid obtained with a small type bronchoscope (Olympus p45) to get BAL fluid from distal parts of the lung where the interaction between the offending pathogens and defending host factors takes place. We found that *M. tuberculosis*, common bacteria, HHV8 and *Pneumocystis jiroveci* were the leading causes of chest infection. Apart from opportunistic infections which capitalize on the lowered innate immunity, this study revealed that common bacterial pathogens of community acquired pneumonia were among the major causes of pulmonary infections even at a very low CD4 T cell counts. Identification of more bacteria may have been hampered by the fact that more that 80% of patients had used antibiotics prior to sampling for the bacteriological studies. PCP was more prevalent than previously reported from this region (3;7), this is likely to occur if appropriately searched for and when different diagnostic tests were used in combination. However the impact of MTB infection in patients infected with HIV was enormous and outweighed all other causes of lung infection: PTB/HIV co-infection was the leading cause of both morbidity and mortality. Its atypical radiological and clinical presentation coupled to poor sputum production and yield resulted into missed or delayed diagnosis and hence opening a window for increased transmission, delayed treatment and poor outcome. Our findings therefore

argue for more aggressive testing strategies for both TB and HIV to increase case detection rate, improve case management, and prevent further transmission.

In **Chapter 4**, we found that HIV infection was associated with high prevalence of the most common human herpes viruses (Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Human herpes virus 8 (HHV8), Herpes simplex virus 1 (HSV1) and Herpes simplex virus 2 (HSV2). Their abundance, irrespective of the other co-infecting pathogens, may imply possible long term negative impact on the outcome of TB/HIV co-infection, given their role in various pathologies e.g. EBV in lymphoproliferative disorders and lymphoma, and HHV8 in Kaposi's sarcoma, Castleman's disease and primary effusion lymphoma (8-13). However, we cannot be totally sure that the HHV DNA demonstrated by PCR in all cases also signified disease due to that virus. Therefore, a larger study also including immunohistochemical stainings of BAL material to identify tissue reactions due to the virus, and longer follow up is warranted to shed more light on their role in TB/HIV co-infection.

As presented in **Chapter 5,** current diagnostic tests are inefficient in TB diagnosis: the WHO recommended AFB smear of sputum for resource-limited settings showed to be limited by very low sensitivity in TB/HIV co-infected patients, even when BAL fluid was used instead of sputum. Other methods, which are either rarely available, expensive, or technically too

demanding showed to have varying sensitivity and specificity. The current C_T cut off value of 40 in real time PCR for MTB resulted into low specificity of this molecular technique; this could be improved by changing the cut off value for the C_T to 32 in TB endemic region. Using the latter cut off value for the PCR applied to BAL material, latent TB may be differentiated from active TB. We recommend however this to be confirmed in a prospective follow-up study in which real time PCR is applied to both sputum and BAL material.

The prevailing high rates of MTB transmission occurring in the region where our studies were done (northern Tanzania), was demonstrated **(Chapter 6)** by the high prevalence of only a couple of genotypes: Cas1-Kili accounted for more than one-third of the genotypes and LAM11–ZWE for more than one-tenth. Predominance of the same genotypes in this region was also previously reported using a different molecular technique (14). More than a third of these patients were HIV co-infected. High MTB transmission rate is associated with HIV infection, on top of poor socio-economical status (15;16). Beijing MTB strain was among the major spoligotypes although it is a recent strain in this region. The fact that MTB Beijing was associated with young age and that it was a major spoligotype was an indication that it is a recent strain and its transmission is ongoing (17).

The upward trend of anti-TB drug resistance we observed could worsen the outcome of TB which is already poor when it is associated with HIV infection. Resistance to INH reached a double digit, MDR and resistance to all first line drugs was higher than previously reported from this country (4). This

indicated an imminent danger of developing extensively drug resistant MTB (XDR), which is associated with both MDR and HIV infection (18). The delay in obtaining MTB drug susceptibility (DST) results and limited correlation between DST and the faster genotypic resistance testing, is a challenge for early identification of treatment non-responders in this part of the world. The delay in identification of non-responders and resistant strains will compound high rates of transmission and poor treatment outcome.

In **Chapter 7**, we reported an immunological study in HIV infected TB patients showing that levels of macrophage migration inhibitory factor (MIF) were higher in HIV infected patients with TB than in non-HIV infected individuals with TB. Unlike in studies without HIV infection (19-21), in the HIV infected TB patients we studied, low MIF levels were associated with poor outcome. These results indicate that the on-going trials on the immunotherapeutic use of anti-MIF therapy to alter fatal outcome associated with bacterial infections in non-HIV infected patients (19;21-25) may not be helpful to the majority of TB patients from sub-Saharan Africa because they are co-infected with HIV. Our study was the first to study MIF levels in BAL and serum of TB/HIV co-infected individuals.

We assayed a broad spectrum of cytokines/chemokines in BAL fluid of patients with TB, strictly defined by TB culture (in which MTB complex was confirmed by DNA probe) and real time PCR for the IS6110 gene, to avoid misdiagnosing TB. We found **(Chapter 8)** that cytokine profiles were

different depending on the radiographical presentation: non-cavitary TB was associated with higher BAL IP 10 and IL 7. These findings apart from being new in the field of TB/HIV also showed that advanced AIDS has a different radiological presentation and an altered immunological response compared to HIV infection with preserved immunity. We also found that chest radiographic features were related to the level of immunity (i.e. CD4 T cell levels): cavitary lesions on chest radiograph were associated with high CD4 T cell count and atypical features appeared at low CD4 T cell count.

As reported in **Chapter 9**, apart from diagnostic problems, also other factors contribute to high morbidity and mortality due to TB in this part of the world. For the Asp299Gly TLR4 polymorphism, which is highly prevalent in sub-Saharan Africa (26) we showed an association with development of active TB; patients with the polymorphism were more likely to develop the disease than those without the polymorphism. We also observed an association between the 299 polymorphism and low CD4 T cell counts, which could as well lead to poor outcome. This looks like a disadvantageous evolutionary shift since the HIV pandemic: from the protective role of Asp299Gly polymorphism against high mortality in malaria (26), to the increased susceptibility to tuberculosis and low CD4 T cell count in TB/HIV co-infection, as we observed in this study. However this hypothesis warrants further studies to explore the interaction of the three diseases (TB, malaria and HIV) since both are chronically present in sub-Saharan Africa.

AVENUES FOR FUTURE RESEARCH

1. To define the role of anti-MIF antibody therapy in a broad range of CD4 T cell counts in TB/HIV co-infected patients. Also, to determine levels of MIF in BAL (with a method for quantification of the obtained BAL material) in additional study groups, particularly TB infected patients without HIV infection.
2. To determine the role of IL-7 in HIV progression in patients co-infected with TB, in order to understand whether it is only a marker of advanced AIDS due to its association with low CD4 T cell count or contributes to HIV pathogenesis through upregulation of RANTES production.
3. To determine the function of IP-10 in TB/HIV co-infection given the apparently dual phenotype of CD4 T cells in TB, both protective (given the increased rates of active TB with CD4 T cell decline) and deleterious (given the association of CD4 cells with cavitary TB and TB burden) observed in our study.
4. To evaluate the long term clinical significance of positive real time PCR for MTB in HIV infected and immunosuppressed patients with negative MTB culture. Also, to study real time PCR in sputum and to optimize the CT cut off value of real time PCR in TB endemic settings to differentiate active TB from latent TB.
5. To determine why Asp299Gly TLR4 polymorphism is highly prevalent in sub-Saharan Africa, despite high susceptibility and mortality associated with TB in its bearers, and to determine in view of the

interaction between TB, HIV and malaria, whether the prevalence of Asp299Gly polymorphism will go down in future in sub-Saharan Africa.

6. To determine the long-term impact of infection with human herpes viruses in TB/HIV co-infection, given their high prevalence and their role in various pathological conditions.

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SUMMARY

This thesis describes clinical and immunological aspects of co-existence in the human host of *Human immunodeficiency virus* (HIV) and *Mycobacterium tuberculosis* (MTB).

Chapter one gives an introduction to the developing HIV epidemic and in its wake the epidemic of tuberculosis (TB) in tropical Africa, elucidating the atypical presentation and diagnostic problems encountered in a resource poor setting. It also highlights research needs in clinical aspects of TB/HIV co-infection and in its pathophysiology and immunology. Further, an outline is given of the research presented in the following chapters of this thesis.

Chapter two on HIV-associated morbidity and mortality among inpatients at a tertiary care referral hospital in northern Tanzania reveals that over a ten-year period there was an incremental trend in hospitalization and mortality attributable to HIV infection. Pulmonary infections were by far the leading causes of morbidity and mortality in both paediatric and adult in-patients. While the contribution of HIV infection to both hospitalization and poor outcome was enormous, 44 % of the patients who tested HIV seropositive in a point-prevalence survey were previously unaware of their infection and were unsuspected clinically as having HIV infection. A plea was made for an improved policy on HIV testing to increase case detection rates, improve case management and prevent spread of the infection.

In **Chapter three** we report a study on aetiology and presentation of AIDS – associated pulmonary infections. A wide spectrum of causative agents was identified. Tuberculosis was common as expected, but prevalence of *Human herpes virus 8* (HHV8) and *Pneumocystis jiroveci* was relatively high for a country in sub-Saharan Africa. An aetiological diagnosis could not be made in more than 40% of patients. These organisms were largely undistinguishable by chest radiographic features, except micronodules, which were significantly more found in tuberculosis. Also we found that even at very low CD4 T cell levels common bacteria were common causes of pneumonia. *M. tuberculosis* and HHV8 were the main causes of death due to pneumonia in HIV infection.

In **Chapter four** data are presented on the prevalence of Human herpes viruses (HHV) in bronchoalveolar lavage (BAL) fluid of two groups of severely immunosuppressed HIV infected patients with pulmonary infections, 25 with pulmonary tuberculosis (TB), and 25 with non-TB pulmonary infections. The groups were matched for age and CD4 T cell count (average 50 cells/µl). We showed that HHV in this part of the world are highly prevalent: Epstein-Barr virus (EBV) was found in 78%, Cytomegalovirus in 24%, HHV8 in 14% and HSV-1 in 14%. HHV were equally prevalent in both, patients with TB and with non-TB lung infections, and HHV prevalence was higher than found in western countries. The difference in mortality between the two groups after one-month follow up was statistically not significant. A study with longer follow up, in a group of patients with a wider range of CD4 T cell counts is

needed to elucidate the pathogenetic role of HHV in HIV infected patients with pulmonary infection.

In **Chapter five** a study is presented on laboratory diagnosis of pulmonary tuberculosis in TB/HIV co-infection and the contribution of real time PCR in the setting of Tanzania. In nearly half of HIV/AIDS patients a microbiological diagnosis of TB could not be made by examination of sputum, as patients either produced no sputum or inadequate sputum. In resource-limited settings sputum is the principle source for the microbiological diagnosis of TB. Even when samples were taken from the BAL compartment, currently available diagnostic techniques are far from optimal in diagnosing TB in HIV co-infected patients. Compared to culture, which is still the gold standard, ZN smear and MycoDot® serological tests had low sensitivity and real time PCR had low specificity. The specificity could however be improved, without significantly affecting its sensitivity, by adopting a lower cycle threshold (C_T) value. The long period to positivity (4 weeks or more) is a limiting factor of culture. We found that during one and a half year of follow up, none of the patients with positive real time PCR but negative culture of BAL fluid developed active TB. Before definite conclusions can be drawn, however, the clinical significance of positive real time PCR in sputum and BAL when culture is negative needs to be studied in a prospective follow up study with longer follow up.

In **Chapter six** results are presented of a study on genotypic diversity and drug susceptibility of *M. tuberculosis* in Tanzanian patients of whom one third was co-infected with HIV. Many different spoligotypes were found, including MTB Beijing, found until recently only in other countries than Tanzania. This implies increased human movement and cross-infections. The dominant spoligotypes were only few and also only few were unique strains, which also implies high transmission rate. We found no or little association between the dominant spoligotypes and anti-TB drug resistance, but identified an upward trend of MTB resistance to commonly used anti-TB drugs, which is a worrisome finding. The percentages of INH resistance and MDR have doubled compared to recent previous reports. This signals that anti-TB drug resistance is an increasing problem. We were also able to show that genotypic drug susceptibility testing though fast, cannot stand by it self to pick clinically resistant strains without phenotypic drug susceptibility testing.

In **Chapter seven** we showed that HIV infection has a major influence on serum macrophage migration inhibitory factor (MIF) levels in HIV seropositive patients from Tanzania with pulmonary infections. Serum MIF levels in HIV infected patients with TB and in HIV infected patients with non-TB pulmonary infections were equally high. Consequently, determination of MIF cannot distinguish TB from other common pulmonary infections in HIV infection. MIF levels in patients with HIV infection were several times higher than in individuals without HIV infection. We also found that the individuals from Tanzania we studied had higher levels of MIF than was found in other

studies from different geographical locations. Unlike previous studies showing an association of high MIF levels with severity of the disease and poor outcome in individuals without HIV infection, we found in our patients from Tanzania that in HIV infection mortality was associated with low MIF levels.

In **Chapter eight** data are presented on interferon γ-inducible protein 10 (IP10) and interleukin-7 (IL 7) in BAL fluid in AIDS-associated tuberculosis. Also the correlation is presented between immune status as expressed by CD4 T cell count and the chest radiographic appearance. Cavitary lesions and large cavities occur when the cellular immunity is fairly preserved. Atypical radiographic features become apparent with deterioration of the immunity. Chest radiographic features are also reflected by the local immune response; patients without cavitary lesions exhibited higher BAL interferon-γ IP 10 and IL 7. Whether IL 7 is a marker of advanced AIDS or contributes to HIV pathogenesis through its up-regulation of RANTES production remains unclear. The major sources of IP 10 and IL7 as shown by immunohistochemistry are BAL neutrophils, monocytes/macrophages, and epithelial cells. We were also able to show that BAL granulocyte macrophage colony-stimulating factor (GM-CSF) was distinctly higher in TB patients than in non-TB patients. This raises argument that if the high levels of GM-CSF are a host protective attempt given the role of GM-CSF in TB containment and granuloma development, then there may be possibilities of immunotherapy using exogenous GM-CSF.

In **Chapter nine** data on Toll- like receptor 4 (TLR4) polymorphism in Africa are presented. Toll-like receptors are important in the host defence against *Mycobacterium tuberculosis.* Also, it has been recently shown that individuals with Asp299Gly polymorphism have a higher malaria parasitaemia, but suffer less from cerebral malaria. In our study, we showed that HIV infected patients in Tanzania carrying the Asp299Gly polymorphism had a higher chance to develop active tuberculosis than HIV infected patients without the polymorphism. We also showed that this could be explained largely by a significant reduction in CD4 T cell level of the patients with the polymorphism. Since Asp299Gly is found in up to a fifth of the population in Africa, this may explain in part why the incidence of tuberculosis in this part of the world is so high. The association of the polymorphism Asp299Gly with low CD4 T cell count may be due to a direct effect of TLR4 receptors on CD4 T cell kinetics.

Thus, the advantage of having the polymorphism for malaria may turn into a disadvantage due to its effect in tuberculosis for patients co-infected with HIV. This is likely to lead to a lower prevalence of this polymorphism in Africa.

In **Chapter ten** the findings reported in the chapters 2 – 9 are discussed and put in perspective of the evolving epidemic of patients with dual infections of HIV and MTB in tropical Africa. Also avenues for future research are explored.

MUHSATARI

Tasnifu hii inaangalia tatizo la ugonjwa wa kifua kikuu unapowapata watu waliopungukiwa na kinga ya mwili kutokana na kuwa na virusi vya UKIMWI.

Sura ya kwanza ni utangulizi unaoonyesha mfumuko wa ugonjwa wa UKIMWI na namna UKIMWI ulivyobadili sura nzima ya ugonjwa wa kifua kikuu katika nchi za Afrika kusini mwa jangwa la Sahara. Sura hii pia inaelezea juu ya utata uliopo katika kuutambua ugonjwa wa kifua kikuu kwa wagonjwa wenye virusi vya UKIMWI. Kisha katika sura hii tafiti zilizomo katika tasnifu hii zinaainishwa.

Sura ya pili inaonyesha ukubwa wa tatizo la ugonjwa wa UKIMWI katika hospitali ya rufaa ya KCMC; ikionyesha ni kiasi gani cha wagonjwa hulazwa hospitalini hapa kwa sababu ya UKIMWI. Sura hii inaonyesha kuwa ugonjwa huu umekuwa ukiongezeka mwaka hadi mwaka kwa kipindi cha miaka kumi mfululizo. Sura hii inaonyesha kwamba magonjwa ya homa ya mapafu kwa ujumla ndiyo sababu kubwa ya kulazwa na hata kufa kwa wagonjwa wenye virusi vya UKIMWI. Pamoja na hayo pia sura hii inaonyesha kwamba bado kiasi cha 44% ya wagonjwa wenye UKIMWI huwa hawatambuliki kwa kuangalia dalili za ugonjwa huo tu. Hivyo yapaswa kutafuta njia mbadala za kuwezesha upimaji wa wagonjwa wengi zaidi ili kupunguza maambukizi na pia kuanza matibabu mbadala mapema.

Sura ya tatu ni taarifa ya utafiti kuhusu mlolongo wa vijidudu vinavyosababisha ugonjwa wa homa ya mapafu. Utafiti unaonyesha kuwa bakteria wa ugonjwa wa kifua kikuu, *Human herpes virus 8* (HHV8), na *Pneumocystis jiroveci* ndivyo vijidudu visababishi vya mara kwa mara vya ugonjwa huu wa homa ya mapafu. Pia utafiti huu unaonyesha kwamba takribani 40% ya wagonjwa hao wa ugonjwa wa homa ya mapafu haikuweza kutambulika vijidudu visababishi vya ugonjwa huo hata baada ya kufanya uchunguzi yakinifu. Utafiti huu unaonyesha kwamba picha za x-ray ya kifua haziwezi kuainisha kwa ukamilifu sababu ya ugonjwa wa homa ya mapafu, ingawa kuwepo kwa *micronodules* kwenye picha ya X-ray ya kifua kunaweza kuhusishwa na ugonjwa wa kifua kikuu. Bakteria wa aina zote kwa ujumla wanaendelea kuwa ndiyo sababu kubwa ya ugonjwa wa homa ya mapafu, na bakteria wa ugonjwa wa kifua kikuu na virusi vya HHV8 ndiyo chanzo kikubwa cha vifo kwa wagonjwa wa UKIMWI.

Sura ya nne inaonyesha kiasi gani cha wagonjwa wa UKIMWI pia wana aina nyingine za virusi kama aina ya *Human herpes viruses*. Kwa kupima maji maji yaliyochukuliwa kutoka kwenye mapafu kwa kutumia Bronkoskopia inaonyesha kuwa tatizo la virusi hivi ni kubwa kwa wagojwa wa UKIMWI katika eneo hili kuliko ilivyo sehemu nyingine duniani. Virusi vya *Epstern Barr, Cytomegalo* na *Human herpes 8* ndivyo vinavyoongoza kwa wingi. Hata hivyo uwepo wa virusi hivi katika utafiti huu haukuweza kuhusishwa na kuwepo kwa bakteria wa ugonjwa wa kifua kikuu kwa wagonjwa hawa wenye upungufu mkubwa wa kinga ya mwili. Pia imeonekana kwamba vifo vya

wagonjwa hawa kwa kipindi cha mwezi mmoja wa kwanza havikuchangiwa na kuwepo kwa vijidudu vya *Human herpes viruses* kwa wagonjwa hawa wa UKIMWI. Hata hivyo ukweli kuhusu madhara ya kuwepo virusi hivi kwa wagonjwa wa UKIMWI na kifua kikuu yanaweza tu kuanishwa kwa uhakika zaidi baada ya utafiti wa muda mrefu zaidi.

Sura ya tano inachambua njia zinazotumika katika kugundua ugonjwa wa kifua kikuu kwa wagonjwa wa UKIMWI. Inaeleza ugumu wa kugundua bakteria wa kifua kikuu (hasa kwa wagonjwa wenye upungufu mkubwa wa kinga ya mwili). Ugumu huo unatokana na shida ya wagonjwa hawa kutoa makohozi, uwezo mdogo wa kipimo cha Ziehl Neelsen wa kutambua bakteria wa kifua kikuu, uwezo mdogo wa MycoDot(R) kuweza kutofautisha bakteria wa kifua kikuu kutoka kwa aina nyingine ya vijidudu kwa wagonjwa wa UKIMWI. Pia utafiti huu unaonyesha kiasi gani kipimo cha kisasa cha "*real time PCR*" kinaelemewa katika kutofautisha ugonjwa kutokana na bakteria wa kifua kikuu au kuwepo tu kwa bakteria hawa bila ugonjwa katika mazingara haya ya kukithiri kwa ugonjwa wa kifua kikuu. Hivyo utafiti huu unashauri kwamba itumike "cut-off ya 32" badala ya "cut-off ya 40" ili kuweza kutofautisha kati ya makundi haya mawili. Kwa sababu wale ambao hawakuonekana kuwa na bakteria hawa kwa njia ya kuotesha (ila kwa PCR tu) hawakuugua ugonjwa wa kikfua kikuu kwa muda wote wa mwaka mmoja na nusu wa kuangaliwa.

Sura ya sita ni utafiti unaoelezea kuhusu jamii ya bakteria wanaounda kundi la *Mycobacterium tuberculosis complex*. Utafiti huu unahusisha aina hizo za bakteria wa kifua kikuu na usugu wao kwa dawa za kutibia ugonjwa wa kifua kikuu. Katika utafiti huu tunaona kwamba kadri ya theruthi moja ya wagonjwa wa kifua kikuu pia wana virusi ya UKIMWI. Tunaona kuwa pamoja na kuwa na aina nyingi za vibakteria vya kifua kikuu, asilimia kubwa ya wagonjwa wana ugua ugonjwa wa kifua kikuu kutokana na aina mbili tu za vijidudu vya jamii ya *Mycobacterium tuberculosis complex,* yaani *Cas 1 Kili na LAM11-Zwe.* Vibakteria vya aina ya Beijing ingawa ni vigeni katika eneo hili vinaonekana kuongezeka. Vibakteria hivi vimekuwa vikihusishwa na usugu kwa dawa za ugonjwa wa kifua kikuu. Kuwepo kwa aina nyingi za bakteria wa jamii ya *Mycobacterium tuberculosis complex* na aina chache tu kuwa ndiyo viambukizi vya mara kwa mara ni dalili ya kuwepo kwa maambukizi mengi na ya mara wa mara katika jamii. Utafiti pia unaonyesha kwamba idadi ya wagonjwa wenye vijidudu sugu kwa dawa za kifua kikuu inaongezeka ukilinganisha na taarifa za awali. Idadi ya wagojwa wenye vijidudu sugu kwa dawa ya Isoniazid na vijidudu vyenye usugu kwa dawa za isoniazid na rifampicin (multidrug resistant strains - MDR) imeongezeka mara dufu ukilinganisha na taarifa za awali. Hii ni dalili ya kukua kwa tatizo hili na huenda ikasababisha usugu wa kupindukia (extremely drug resistant – XDR). Tatizo la kuweza kugundua vijidudu sugu kwa muda muafaka bado halijapata utatuzi kwa sababu ya kuchukua muda mrefu kuviotesha vijidud vya kifua kikuu, na pia bado uhusiano wake na ule wa njia ya *genotyping* bado hauna uhakika wa kutambua kwa usahihi vijidudu sugu.

Sura ya saba inaelezea juu ya tofauti kati ya wagonjwa wenye virusi vya UKIMWI ukilingalisha na wale wasiokuwa na virusi hivyo. Tofauti hizo zilitokana na kwamba virusi vya UKIMWI vinatawala kwa kiasi kikubwa mgongano kati ya kinga ya binadamu na vijidudu vya ugojwa. Utafiti huu umeonyesha kwamba kiwango cha *Macrophage migration inhibitory factor* (MIF) kinakuwa juu sana kwa wagonjwa wa UKIMWI ukilinganisha na wale wasio na ugonjwa huo. Pia tunaona kuwa tofauti ni kwamba wale waliokufa kutokana na ugonjwa wa kifua kikuu walikuwa na kiwango cha chini cha MIF ukilinganisha na waliopona. Hii ni tofauti Kubwa ukilinganisha na wagonjwa wenye kifua kikuu bila ya kuwa na UKIMWI; kwa wale wasio na UKIMWI wanaokufa huwa na kiwango cha juu sana cha MIF kuliko wale wanaopona.

Sura ya nane inaonyesha kwamba kwa wagonjwa wa kifua kikuu kiwango cha kupungua kwa kinga ya mwili kinaambatana na mabadiliko katika picha za X-ray ya kifua. Wale wanaokuwa na matundu katika picha zao za x-ray kinga yao ya mwili huwa angalao ni nzuri kidogo kuliko wale ambao huwa hawana matundu katika x-ray. Pia inaonyesha kwamba wale ambao hawana matundu huwa na kiwango kikubwa cha IP10 na IL 7 kuliko wale wenye matundu. Hivyo hivyo wale wenye ugonjwa wa kifua kikuu huwa na kiwango kikubwa cha GM-CSF kuliko wale ambao hawana ugonjwa wa kifua kikuu. Hii yote inaonyesha kiasi gani ugonjwa huu wa UKIMWI umebadili sura nzima ya magonjwa mengine.

Sura ya tisa inaelezea mabadiliko katika Toll – like receptor 4 (TLR4) Asp299Gly ambayo yametokea kwa binadamu baada ya miaka mingi ya kupambana na ugonjwa wa malaria yakiwa na lengo la kumkinga binadamu na ugonjwa huo, lakini sasa yanaonekana kuchangia kuugua ugonjwa wa kifua kikuu na kupungua kwa kinga ya mwili kwa wagonjwa wenye viruis vya UKIMWI. Hivyo basi madiliko haya ambayo mwanzoni yalikuwa ya manufaa kwa binadamu sasa yanaonekana kuwa na madhara kwa sababu ya kuwepo kwa ugonjwa huu wa UKIMWI.

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SAMENVATTING

In dit proefschrift worden klinische en immunologische aspecten beschreven van gelijktijdige infectie met *Human immunodeficiency virus* (HIV) en *Mycobacterium tuberculosis* (MTB) bij de mens.

Hoofdstuk 1 geeft een introductie op de ontwikkeling van de HIV-epidemie en de erop volgende epidemie van tuberculose (TB) in tropisch Afrika, waarbij de atypische presentatie en de diagnostische problemen in ontwikkelingslanden worden toegelicht. Ook wordt uiteengezet op welke gebieden wetenschappelijk onderzoek nodig is t.a.v. klinische aspecten van TB/HIV co-infectie, alsook t.a.v. van de pathofysiologische en immunologische kanten van deze dubbelinfectie. Dit hoofdstuk geeft ook een overzicht van de verschillende studies die in de erop volgende hoofdstukken van het proefschrift worden gepresenteerd.

Hoofdstuk 2 beschrijft de met HIV geassocieerde morbiditeit en mortaliteit bij patiënten opgenomen op een derdelijnsziekenhuis in noord Tanzania. Over een periode van 10 jaar werd een toename gezien in de ziekenhuisopnames en de sterfte ten gevolge van HIV-infectie. Longinfecties waren verreweg de belangrijkste oorzaak van morbiditeit en mortaliteit, zowel bij volwassenen als kinderen. De bijdrage van HIV-infectie aan zowel ziekenhuisopname als sterfte was indrukwekkend, en 44 % van de patiënten die HIV-seropositief waren in een prevalentieonderzoek, waren zich tevoren niet bewust van hun HIV-infectie en deze werd ook niet vermoed door de

behandelende artsen. Gepleit wordt voor een beter testbeleid zodat meer gevallen van HIV-infectie kunnen worden opgespoord, de patiënten beter worden behandeld en verdere verspreiding van HIV-infectie wordt tegengegaan.

Hoofdstuk 3 beschrijft een onderzoek naar verwekkers en klinische presentatie van met AIDS geassocieerde longinfecties, waarbij een grote variëteit van ziekteverwekkers werd geïdentificeerd. Zoals verwacht kwam tuberculose veel voor, maar ook *Humaan herpes virus 8* (HHV8) en *Pneumocystis jiroveci,* zeker voor een land in Afrika ten zuiden van de Sahara. Een oorzakelijke diagnose werd niet gesteld bij meer dan 40% van de patiënten. De gevonden ziekteverwekkers konden op basis van de röntgenfoto van de longen vrijwel niet van elkaar worden onderscheiden. Wel werden micronodulaire afwijkingen significant meer gezien bij tuberculose. Ook werd vastgesteld dat, zelfs bij zeer lage waarden van het aantal CD4 T–cellen, gewone bacteriën vaak de verwekkers van longontsteking waren. Tuberculose en infectie met HHV8 waren de belangrijkste doodsoorzaken bij met HIV geïnfecteerde patiënten met een longontsteking.

In **hoofdstuk 4** worden gegevens gepresenteerd over de prevalentie van Humane herpes virussen (HHV) in bronchoalveolaire lavage (BAL) vloeistof bij twee groepen met HIV geïnfecteerde patiënten met ernstige immunosuppressie en tekenen van longinfectie: 25 met longtuberculose, en 25 met een niet-tuberculeuse longinfectie. De

groepen waren gematched voor leeftijd en aantal CD4 T-cellen (gemiddeld 50 cellen/µl). HHV infectie kwam zeer veel voor in dit deel van de wereld: Epstein-Barr virus (EBV) werd gevonden bij 78%, Cytomegalovirus bij 24%, HHV8 bij 14% en HSV-1 bij 14%. De prevalentie van HHV bij de patiënten met longtuberculose was gelijk aan die bij de patiënten met niet-tuberculeuse longinfecties en hoger dan die in westerse landen. Het verschil in sterfte tussen de twee groepen een maand na opname in de studie was statistisch niet significant. Om de rol die HHV spelen bij met HIV geïnfecteerde patiënten met longinfectie op te helderen, wordt gepleit voor een onderzoek waarbij patiënten met een grotere variatie in CD4 T-cellen gedurende langere tijd worden vervolgd.

In **hoofdstuk 5** wordt een onderzoek gepresenteerd naar de laboratoriumdiagnose van longtuberculose bij patiënten met HIV-infectie, en de bijdrage van real time PCR (rtPCR) hierbij, in de vigerende omstandigheden van Tanzania. Bij bijna de helft van de patiënten kon de microbiologische diagnose van tuberculose (TB) niet worden gesteld door sputumonderzoek, omdat de patiënten geen of inadequaat sputum produceerden. In arme landen is sputum de belangrijkste bron voor de microbiologische diagnose van longtuberculose. Zelfs als bronchoalveolaire lavage (BAL) vloeistof

wordt gebruikt zijn de momenteel beschikbare diagnostische technieken verre van optimaal om longtuberculose vast te stellen bij TB patiënten met HIV-infectie. Vergeleken met kweek als de standaard methode, bleek onderzoek op zuurvaste staven (ZN) alsook de serologische MycoDot® test, slecht te voldoen door hun lage gevoeligheid, terwijl de rtPCR een lage specificiteit had. De specificiteit van de rtPCR werd beter, zonder significant verlies aan sensitiviteit, als een lagere cycle threshold (C_T) waarde werd gebruikt. Voor de TB kweek is de lange tijd (4 weken of meer) die nodig is voordat deze positief wordt, een beperkende factor. In ons onderzoek vonden wij dat geen enkele van de patiënten met positieve rtPCR maar negatieve TB kweek van BAL vloeistof, en die gedurende anderhalf jaar werden vervolgd, actieve TB ontwikkelden. Om een definitieve conclusie te trekken over de klinische betekenis van een positieve uitslag van de rtPCR, moet eerst een prospectief vervolgonderzoek worden gedaan waarbij de rtPCR wordt geëvalueerd in sputum en BAL bij patiënten met een negatieve TB kweek en die langer worden vervolgd dan in het huidige onderzoek gebeurde.

In **hoofdstuk 6** worden de resultaten gepresenteerd van een onderzoek naar de genotypische diversiteit en de geneesmiddelengevoeligheid van 130 *M. tuberculosis* (MTB) isolaten

van Tanzaniaanse patiënten, waarvan eenderde een co-infectie met HIV had. Zevenenveertig verschillende spoligotypen werden gevonden, inclusief MTB Beijing, een type dat tot voor kort alleen buiten Tanzania was gevonden. Dit duidt op toegenomen menselijke migratie en kruisinfecties. De meeste spoligotypen (106/130) behoorden tot slechts 9 phylogenetische groepen. Er werden ook enkele unieke stammen gevonden, hetgeen wijst op hoge transmissie. Wij vonden geen duidelijke associatie tussen de dominante spoligotypen en resistentie tegen eerstelijnstuberculostatica. Wel leek een zorgwekkende toename te bestaan in het voorkomen van resistentie tegen tuberculostatica. Het percentage van INH-resistentie en dat van resistentie tegen meerdere geneesmiddelen (MDR stammen) was verdubbeld in vergelijking met vroeger gepubliceerde gegevens. Dit wijst erop dat resistentie tegen tuberculostatica een toenemend probleem is. Ons onderzoek liet ook zien dat genotypisch onderzoek om resistentie tegen tuberculostatica op te sporen weliswaar weinig tijd kost, echter op zichzelf ongeschikt is om klinisch relevante resistentie van MTB stammen op te sporen. Hiervoor blijven de bestaande fenotypische resistentietesten belangrijk.

In **hoofdstuk 7** tonen wij aan dat HIV-infectie in belangrijke mate de serumwaarden van macrophage migration inhibitory factor (MIF)

beïnvloedt bij patiënten met HIV en verschijnselen van longinfectie in Tanzania. De waarden van MIF in het serum van patiënten met HIV-infectie en tuberculose (TB) waren even hoog als de MIF-waarden bij patiënten met HIV-infectie die niet-tuberculeuze longinfecties hadden. Door bepaling van MIF kan zodoende geen onderscheid gemaakt worden tussen longtuberculose en longinfecties van andere aard bij met HIV geïnfecteerde patiënten. De waarden van MIF bij patiënten met HIV-infectie waren aanzienlijk hoger dan bij mensen zonder HIV-infectie. Ook vonden wij dat bij de personen uit Tanzania die wij onderzochten de MIF-waarden hoger waren dan de waarden die door onderzoekers uit andere delen van de wereld werden gevonden. Wij vonden, in tegenstelling tot de bevindingen van anderen bij mensen zonder HIV-infectie, dat bij onze Tanzaniaanse patiënten met HIV-infectie, sterfte was geassocieerd met lage MIF-waarden.

In **hoofdstuk 8** worden gegevens gepresenteerd over interferon γ-inducible protein 10 (IP10) en interleukin-7 (IL 7) in BAL-vloeistof van patiënten met tuberculose (TB) en AIDS. Ook wordt de correlatie gepresenteerd tussen het niveau van de immuniteit, gemeten aan de waarde van het aantal CD4 T-cellen, en de bevindingen op de röntgenfoto van de thorax. Holtevorming als zodanig, alsook grote holten, werden gezien als de cellulaire immuniteit nog redelijk goed

was, terwijl atypische beelden op de thoraxfoto werden gezien als de immuniteit ernstig gestoord was. De röntgenologische presentatie vindt ook zijn reflectie in de lokale immuunrespons: patiënten zonder holtevorming hadden hogere interferon-γ, IP 10 en IL 7 waarden in de BAL-vloeistof. Of IL 7 een marker is voor AIDS in een laat stadium van de ziekte, of bijdraagt aan de pathogenese van HIV via een hogere RANTES productie, is nog onduidelijk. Immunohistochemisch onderzoek van BAL-materiaal liet zien dat neutrophielen, monocyten / macrophagen en epitheelcellen de belangrijkste bron voor IP 10 en IL7 zijn. Ook toonden wij aan dat granulocyte macrophage colony-stimulating factor (GM-CSF) in BAL duidelijk hoger was bij TB patiënten dan bij patiënten zonder TB. Dit is een argument voor de stelling dat hoge GM-CSF waarden een beschermingsmechanisme van de gastheer vertegenwoordigen. Als inderdaad GM-CSF bij tuberculose een functie heeft om de ziekte binnen de perken te houden en de ontwikkeling van granulomata te bevorderen, opent dit mogelijkheden om exogeen GM-CSF te gebruiken voor immunotherapie bij tuberculose.

In **hoofdstuk 9** worden de uitkomsten gepresenteerd van een onderzoek naar Toll- like receptor 4 (TLR4) polymorfisme in Tanzania. Toll-like receptors spelen een belangrijke rol bij de afweer door de

gastheer tegen *Mycobacterium tuberculosis.* Recent onderzoek liet zien dat personen met een Asp299Gly polymorfisme een hogere malaria-parasitemie hebben, echter minder cerebrale malaria. In ons onderzoek toonden wij aan dat met HIV geïnfecteerde patiënten in Tanzania die het Asp299Gly polymorfisme hadden, een grotere kans hebben op het krijgen van actieve tuberculose dan HIV-geïnfecteerde patiënten zonder dit polymorfisme. Wij lieten ook zien dat dit voor het grootste deel wordt veroorzaakt door een significant lager aantal CD4 T-cellen bij de patiënten met het polymorfisme. Omdat het Asp299Gly polymorfisme voorkomt bij ongeveer een vijfde van de bevolking van Afrika, kan hiermee verklaard worden waarom tuberculose zoveel voorkomt in dit deel van de wereld. De associatie van het Asp299Gly polymorfisme met een laag aantal CD4 T-cellen kan het gevolg zijn van een direct effect van de TLR4 receptoren op de kinetiek van de CD4 T-cellen.

Aldus kan het oorspronkelijke voordeel van het polymorfisme ten aanzien van malaria overgaan in een nadeel door het negatieve effect bij patiënten met tuberculose die met HIV geïnfecteerd zijn. Hierdoor zal waarschijnlijk in de toekomst de prevalentie van dit polymorfisme in Afrika gaan dalen.

In **hoofdstuk 10** worden de bevindingen zoals gerapporteerd in de hoofdstukken 2 – 9 besproken en geplaatst in het perspectief van de zich ontwikkelende epidemie van dubbelinfecties met HIV en *M. tuberculosis* bij patiënten in tropisch Afrika. Ook worden doelstellingen aangegeven voor toekomstig wetenschappelijk onderzoek op dit terrein.

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Curriculum vitae (CV)

The writer of this thesis was born in Njombe, Iringa, Tanzania. He studied medicine in Bulgaria, where he was awarded the MD degree from Pleven University of Medicine in 1996. He was trained in bronchoscopy and infectious diseases at Cologne University Hospital, Germany in 1998, and in advanced gastrointestinal endoscopy and bronchoscopy in The Netherlands in 2001 and 2003, and in The USA in 2004. He specialized in Internal Medicine at Kilimanjaro Christian Medical Centre (KCMC), Moshi, Tanzania, where he was awarded the degree of Master of Medicine (MMed) from KCM College, Tumaini University in 2003. Shortly thereafter, he started the research in pulmonary tuberculosis and Human Immunodeficiency Virus co-infection, which led to the present PhD thesis. Currently he is a consultant physician / endoscopist at KCMC, and a Lecturer at KCM College of Tumaini University. He is a father of two children.